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CONTENTS

No. 1, JANUARY—FEBRUARY, 1959

EDITORIAL VIEWS AND NEWS ---------- 1

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. Announcement of 1959 General Meeting ---------- 2

NITROGEN CONTENT OF ZONES DISTINGUISHED BY REFLECTED LIGHT IN ENAMEL SECTIONS FROM UNERUPTED THIRD MOLARS. *L. W. Wachtel, Lieutenant Commander, MSC, USNR,* Bethesda, Md. ---------- 3

SCATTER RADIATION EMANATING FROM THE PATIENT DURING DENTAL ROENTGENOGRAPHIC PROCEDURES. *Duane W. Lovett,* Iowa City, Iowa ---------- 9

A COMPARATIVE HISTOLOGIC STUDY OF HUMAN PERIODONTAL STRUCTURES UTILIZING FLUORESCENCE AND VISIBLE LIGHT MICROSCOPY. *Alvin C. Hileman,* Columbus, Ohio 15

THE EFFECTS OF BETA-AMINOPROPIONITRILE UPON THE MOLAR TEETH OF THE RAT. *George A. Krikos,* Rochester, N. Y. ---------- 27

RADIATION OUTPUT OF DENTAL X-RAY UNITS. *Duane W. Lovett,* Iowa City, Iowa ---- 36

THE EFFECT OF pH ON THE ANTIMICROBIAL ACTIVITY OF PARA-AMINOTOLUENE SULFONAMIDE. *B. F. Gurney,* Chicago, Ill. ---------- 42

A COMPARISON OF BONE GROWTH IN NORMAL AND STRONTIUM-TREATED RATS. *William B. Savchuck,* Bethesda, Md. ---------- 49

THE POLARIZED LIGHT APPEARANCES OF HUMAN DENTAL ENAMEL. *J. H. Allan,* Sheffield, England ---------- 60

THE EFFECT OF CASTRATION ON THE SUBMAXILLARY GLAND OF HUNT-HOPPERT CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS. *Leo M. Sreebny, Sam Rosen, Erica Bachem, H. R. Hunt, and C. A. Hoppert,* Seattle, Wash. ---------- 67

THE EFFECT OF VITAMIN A DEFICIENCY UPON EXPERIMENTAL ORAL CARCINOGENESIS. *Nathaniel H. Rowe and Robert J. Gorlin,* Minneapolis, Minn. ---------- 72

HALF THICKNESS OF DENTIN TO P^{32} BETA RADIATION. *Takao Fusayama, Kotaro Watanabe, and Toshimasa Inaba,* Bunkyo-ku, Tokyo, Japan ---------- 84

ANTIBACTERIAL ACTION OF CERTAIN FLUORIDE-CONTAINING DENTAL RESTORATIVE MATERIALS. *Sudarshan L. Mangi, Wm. A. Summers, Ralph W. Phillips, and Marjorie L. Swartz,* Indianapolis, Ind. ---------- 88

THE ROLE OF ACIDITY OF SALIVA IN THE NUTRITION OF ORAL ACIDOGENS. *L. H. Lightfoot and T. B. Coolidge,* Chicago, Ill. ---------- 96

BACTERIOLOGIC STATUS OF PERIAPICAL TISSUE IN 150 CASES OF INFECTED PULPLESS TEETH. *Louis I. Grossman,* Philadelphia, Pa. ---------- 101

DIFFERENCES IN THE MORPHOLOGY AND SIZE OF THE TEETH OF A CARIES-SUSCEPTIBLE AND A CARIES-RESISTANT STRAIN OF RATS. *R. M. Grainger, K. J. Paynter, and J. H. Shaw,* Boston, Mass. ---------- 105

SALIVARY GLAND FUNCTION IN THE RAT. III. PROTEASE AND ARGINASE ACTIVITY OF SUBMAXILLARY GLANDS AND WHOLE SALIVA. *William G. Shafer, Patricia G. Clark, and Joseph C. Muhler,* Indianapolis, Ind. ---------- 121

FLUORIDE CONTENT OF HUMAN TEETH FROM THE ORIENT AND THE CANADIAN ARCTIC. *James H. Shaw, Joan B. Resnick, and Edward A. Sweeney,* Boston, Mass. ------ 129

VARIABILITY OF TOOTH FORMATION. *Stanley M. Garn, Arthur B. Lewis, and Demarest L. Polacheck,* Yellow Springs, Ohio ---------- 135

ANALYSIS OF AN ANOMALOUS OCULODENTOFACIAL PATTERN IN NEWBORN RATS PRODUCED BY MATERNAL HYPERVITAMINOSIS A. *Frederick M. Deuschle, Jean F. Geiger, and Josef Warkany,* Cincinnati, Ohio ---------- 149

EFFECT OF REPEATED ADDITIONS OF SUBOPTIMUM AMOUNTS OF A REQUIRED VITAMIN ON GROWTH AND ACID PRODUCTION OF LACTOBACILLI. *Stewart A. Koser and Joan Thomas Stuedell,* Chicago, Ill. ---------- 156

STREPTOCOCCI IN DENTAL CARIES. *J. Yardeni, D. Sulitzeanu, and N. Citri,* Jerusalem, Israel ---------- 164

TIME COURSE OF PAROTID SECRETION IN MAN. *L. H. Schneyer, William J. Du Bose, L. K. Levin, and Harry E. Ohme III,* Birmingham, Ala. ---------- 172

STUDIES ON SALIVARIADENECTOMIZED HUNT-HOPPERT CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS. II. INFLUENCE OF SALIVARIADENECTOMY ON RATE OF FOOD CONSUMPTION. *S. Rosen, L. M. Sreebny, H. R. Hunt, C. A. Hoppert, and E. Bachem,* Chicago, Ill. ---------- 177

ELECTRON MICROSCOPY OF DEVELOPING ENAMEL MATRIX IN THE SYRIAN HAMSTER. *Mervyn B. Quigley,* Birmingham, Ala. ---------- 180

A METHOD OF MEASURING THE ADHESION OF FOODSTUFFS TO TOOTH SURFACES. *Robert C. Caldwell,* Birmingham, Ala. ---------- 188

RESEARCH ANNOTATION: DEVELOPMENT OF COATINGS FOR APPLICATION TO TEETH. *Robert C. Caldwell, Anne Gallagher, and R. Winston Liggett,* Birmingham, Ala. 197

RESEARCH ANNOTATION: LYMPHATIC DRAINAGE OF TEETH IN DOGS DEMONSTRATED BY RADIOACTIVE COLLOIDAL GOLD. *Leon Kraintz, Charles D. Tyler, and Benton R. Ellis,* Houston, Texas ---------- 198

RESEARCH ANNOTATION: A METHOD FOR PRODUCING FLUORIDE-FREE SALTS AND A FLUORIDE-FREE DIET. *J. F. McClendon,* Philadelphia, Pa. ---------- 199

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH: Proceedings of Japanese Section. 200

No. 2, MARCH—APRIL, 1959

EDITORIAL VIEWS AND NEWS ---------- 207

THE INFLUENCE OF THE STAGE OF TOOTH DEVELOPMENT ON THE OXYGEN QUOTIENT OF NORMAL BOVINE DENTAL PULP. *Alton K. Fisher, James H. Belding, Jack S. Opinsky, and Daniel J. Spinella,* Iowa City, Iowa ---------- 208

A DESCRIPTION OF THE MOLAR TEETH AND INVESTING TISSUES OF NORMAL GUINEA PIGS. *A. M. Hunt,* Toronto, Ontario, Canada ---------- 216

THE EFFECTS OF ASCORBIC ACID DEFICIENCY ON THE TEETH AND PERIODONTAL TISSUES OF GUINEA PIGS. *A. M. Hunt and K. J. Paynter,* Toronto, Ontario, Canada ---- 232

BLOOD FLOW AND BLOOD PRESSURE IN THE MANDIBULAR ARTERY. *Jack G. Bishop, J. Lester Matthews, Homer L. Dorman, and E. Earl Moore,* Dallas, Texas -------- 244

CALCIFICATION. XXII. A METHOD OF STUDYING CRYSTAL GROWTH. *Joseph Samachson, Sidney Nobel, and Albert E. Sobel,* Brooklyn, N. Y. ---------- 253

A BACTERIOLYTIC AGENT IN SALIVARY GLOBULIN OF CARIES-IMMUNE HUMAN BEINGS. *Gordon E. Green,* Columbus, Ohio ---------- 262

BLOOD FLOW AND BLOOD PRESSURE RESPONSES TO MANDIBULAR NERVE BLOCKING AGENTS. *J. L. Matthews, J. G. Bishop, E. E. Moore, and H. L. Dorman,* Dallas, Texas ---------- 276

SALIVARY OXIDATION-REDUCTION POTENTIALS. *D. H. Norman and G. H. Tucker,* Middlesex, England ---------- 281

TRACE ELEMENTS IN ANCIENT INDIAN TEETH. *L. T. Steadman, F. Brudevold, F. A. Smith, D. E. Gardner, and M. F. Little,* Rochester, N. Y. ---------- 285

CHANGES IN MICROHARDNESS OF ENAMEL FOLLOWING TREATMENT WITH LACTATE BUFFER. *Ernest Newbrun, Philip Timberlake, and Ward Pigman,* Birmingham, Ala. 293

THE EFFECT OF LOCAL X-RAY IRRADIATION UPON THE TEETH OF THE ADULT AXOLOTL (SIREDON MEXICANUM). *V. V. Brunst,* Buffalo, N. Y. 301

A SELECTIVE MEDIUM FOR COUNTING FUSIFORM ORGANISMS IN DENTAL SPECIMENS. *Masao Onisi,* Tokyo, Japan 311

AMINO ACID COMPOSITION OF PROTEINS OF HUMAN DENTIN. *Hubert H. Hughston, Lewis S. Earle, and Francis Binkley,* Atlanta, Ga. 323

THE RESPIRATION OF THE SALIVARY MICROBIAL FLORA AND ITS RELATION TO DENTAL CARIES ACTIVITY. *George H. Green, John H. Kay, and Joseph C. Calandra,* Great Lakes, Ill., and Chicago, Ill. 328

HEREDITY AND DENTAL CARIES. *J. N. Mansbridge,* Edinburgh, Scotland 337

A METHOD FOR DETERMINING THE EFFICIENCY OF LOCAL ANESTHESIA. *George J. Petros and Joseph A. Gibilisco,* Rochester, Minn. 348

INHIBITION OF GLYCOLYSIS BY HALOGENATED COMPOUNDS. *R. S. Manly, Paul H. Doleman, Gladys Hargreaves, and Robert Pillard,* Boston, Mass. 356

CHEMICAL AND PHYSICAL STUDIES OF ENAMEL FROM HUMAN TEETH. IV. DENSITY AND NITROGEN CONTENT OF MOTTLED ENAMEL. *B. R. Bhussry,* Rochester, N. Y. 369

PAROTIN AND GROWTH CENTERS OF FEMURS AND INCISORS IN MICE. *Harold S. Fleming,* Washington, D. C. 374

ELECTRON MICROSCOPE STUDIES OF THE SUBMICROSCOPIC STRUCTURE OF THE DENTINAL MATRIX. *Douglas M. Hall,* Lower Hutt, New Zealand 386

REMOVAL OF RADIOACTIVE FAT AND PROTEIN FROM THE MOUTH. *W. W. Wainwright, F. K. Bauer, and P. B. Thomas,* Los Angeles, Calif. 392

ORAL CHANGES IN NORMAL AND SCORBUTIC GUINEA PIGS INJECTED WITH ASCORBIC ACID-1-C^{14}. *Seymour H. Yale, Henry Jeffay, Clive I. Mohammed, and Edward C. Wach,* Chicago, Ill., and San Juan, Puerto Rico 396

RESEARCH ANNOTATION: RELATIONSHIP OF CARIES INCIDENCE IN DECIDUOUS AND PERMANENT DENTITIONS. *P. Bruszt,* Baja, Hungary 416

RESEARCH ANNOTATION: COLLECTION AND STERILIZATION OF SUBMAXILLARY-SUBLINGUAL SALIVA. *Frederick M. Parkins and Ned B. Williams,* Philadelphia, Pa. 417

RESEARCH ANNOTATION: EFFECT OF RATE OF FREEZING ON THE PRESERVATION OF VIABLE TOOTH GERMS. *Robert Gerstner and Earl O. Butcher,* New York, N. Y. 418

RESEARCH ANNOTATION: A CAPILLARY NETWORK IN THE ODONTOBLASTIC LAYER OF DEVELOPING TEETH. *J. Klingsberg, Louis Cancellaro, and E. O. Butcher,* New York, N. Y. 419

RESEARCH ANNOTATION: EFFECTS OF EXTERNAL ENVIRONMENT ON MUCOSA OF THE HAMSTER'S POUCH. *Earl O. Butcher and Percy L. Johnson,* New York, N. Y. 420

RESEARCH ANNOTATION: AGING, DIET, AND PERIODONTAL LESIONS IN THE HAMSTER. *J. Klingsberg and E. O. Butcher,* New York, N. Y. 421

RESEARCH ANNOTATION: EFFECTS OF DIFFERENT PRESERVATIVES AND SILICONE DENTIFRICES ON CONTACT ANGLE OF WATER ON TOOTH ENAMEL. *Bhim S. Savara, Nilkanth M. Phatak, Jack DeMent, Barbara Schmaling, and Mary Krakowski,* Portland, Ore. 422

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH: SECTIONAL MEETINGS 423

No. 3, MAY—JUNE, 1959

SUPPORTING ASSOCIATES FOR 1959 425

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. Announcement of 1960 General Meeting 426

EDITORIAL VIEWS AND NEWS 427

AUTORADIOGRAPHS OF SOLDER JOINTS WITH FLUORINE.[18] *Saul Bien and Herbert D. Ayers, Jr.*, New York, N. Y. ---- 428

THE DISTRIBUTION OF NITROGEN IN HUMAN ENAMEL. *Ardis Savory and Finn Brudevold*, Rochester, N. Y. ---- 436

DISTRIBUTION OF SOME ENZYMES IN SOFT TISSUES OF THE ORAL CAVITY. *O. Svoboda, Z. Lojda, and M. Skach*, Prague, Czechoslovakia ---- 443

DENTAL CONDITIONS OF PRESCHOOL CHILDREN (ONE TO SIX YEARS OF AGE) IN SZEGED, HUNGARY. *K. Toth and I. Szabó*, Szeged, Hungary ---- 451

COMPARATIVE EFFECTS OF FLUORIDES ON MICE FEMURS. *Harold S. Fleming*, Washington, D. C. ---- 464

THE RELATIVE LOCAL ANESTHETIC POTENCIES OF PROCAINE, ORACAINE, KINCAINE, AND XYLOCAINE. *Stanley C. Harris and Robert C. Worley*, Chicago, Ill. ---- 477

PHASE COMPOSITION OF CROWN AND INLAY PORCELAINS. *Jean T. Hodson*, Seattle, Wash. ---- 483

STUDY OF THE SETTING OF PLASTER. *Knud Dreyer Jørgensen and Aaron S. Posner*, Washington, D. C. ---- 491

PHOSPHATASE ACTIVITY IN WHOLE AND PAROTID SALIVA AND ITS RELATIONSHIP TO DENTAL CARIES. *Shoji Saito and Kozi Kizu*, Tokyo, Japan ---- 500

A SOURCE OF ERROR IN ALKALINITY TITRATIONS OF SALIVA. *Poul Grøn*, Boston, Mass. 506

OBSERVATIONS ON THE DEVELOPMENT OF OXYTALAN FIBERS IN THE PERIODONTIUM OF MAN. *Harold M. Fullmer*, Bethesda, Md. ---- 510

THE EFFECT OF PEPTIDES AND AMINO ACID DERIVATIVES ON ACID FORMATION IN SUGAR-SALIVA MIXTURES. *L. S. Fosdick and R. Quentin Blackwell*, Chicago, Ill. 519

DENTAL CARIES IN THE SYRIAN HAMSTER. VIII. THE INDUCTION OF RAMPANT CARIES ACTIVITY IN ALBINO AND GOLDEN ANIMALS. *Paul H. Keyes*, Bethesda, Md. ---- 525

SUTURAL EXPANSION BY CONTROLLED MECHANICAL STRESS IN THE RAT. *Charles J. Burstone and William G. Shafer*, Indianapolis, Ind. ---- 534

DECOMPOSITION OF GYPSUM INVESTMENT IN THE PRESENCE OF CARBON. *William J. O'Brien and John P. Nielsen*, New York, N. Y. ---- 541

URINARY FLUORINE LEVELS IN WOMEN DURING PREGNANCY AND AFTER DELIVERY. *Itzhak Gedalia, Aron Brzezinski, and Bruno Bercovici*, Jerusalem, Israel ---- 548

HISTOLOGIC OBSERVATIONS ON THE MORPHOLOGY OF THE BLOOD VESSELS OF CANINE AND HUMAN TOOTH PULP. *Thomas C. Cheng and D. Vincent Provenza*, Baltimore, Md. 552

ELECTRON MICROSCOPY OF THE AMELODENTINAL JUNCTION DURING EARLY DEVELOPMENT OF THE MOLARS OF HAMSTERS. *Mervyn B. Quigley*, Birmingham, Ala. ---- 558

THE DIFFUSION OF GLUCOSE THROUGH MICROBIAL PLAQUES. *C. Huh, R. Q. Blackwell, and L. S. Fosdick*, Chicago, Ill. ---- 569

THE BW1081 MOUSE SALIVARY GLAND TUMOR. *James O. Beck, Jr.*, Indianapolis, Ind. 577

STUDIES OF SALIVARIADENECTOMIZED HUNT-HOPPERT CARIES-RESISTANT RATS. III. EFFECT OF SALIVARIADENECTOMY ON DENTAL CARIES AND SEVERAL GROUPS OF THE ORAL MICROFLORA UNDER CONTROLLED FEEDING CONDITIONS. *S. Rosen, L. M. Sreebny, C. A. Hoppert, H. R. Hunt, and E. Bachem*, Chicago, Ill. ---- 587

HISTOCHEMICAL ANALYSIS OF LESIONS IN INCIPIENT DENTAL CARIES. *Ralph R. Steinman, C. Gordon Hewes, and Robert W. Woods*, Loma Linda, Calif. ---- 592

THE EFFECT OF VARYING PEROXIDE AND POLYMER ON THE RATE AND DEGREE OF POLYMERIZATION OF POLYMETHYL METHACRYLATE SLURRIES. *John A. Cornell and Catherine M. Powers*, Philadelphia, Pa. ---- 606

A SURVEY OF LACTOBACILLI INCLUDING PIGMENTED STRAINS FROM THE ORAL CAVITY OF THE WHITE RAT. *H. V. Jordan, R. J. Fitzgerald, and J. E. Faber, Jr.*, Bethesda, Md., and College Park, Md. ---- 611

RELATIONSHIP BETWEEN MATERNAL INGESTION OF CARBOHYDRATE IN THE RAT AND DENTAL CARIES SUSCEPTIBILITY OF THE PROGENY. *Benjamin F. Watson and Joseph C. Muhler*, Indianapolis, Ind. ---- 618

RELATION OF STRUCTURE TO THE MICROHARDNESS OF HUMAN DENTIN. *R. G. Craig, P. E. Gehring, and F. A. Peyton*, Ann Arbor, Mich. ---- 624

RESEARCH ANNOTATION: AGE VARIATIONS IN THE NUMBER OF MAST CELLS IN ORAL MUCOSA AND SKIN OF ALBINO RATS. *F. A. Carranza, Jr., and R. L. Cabrini*, Buenos Aires, Argentina ---- 631

RESEARCH ANNOTATION: INCIDENCE AND DISTRIBUTION OF ORAL PLEUROPNEUMONIA-LIKE AND L ORGANISMS. *George W. Burnett and Eleanor Gilmore*, Washington, D. C. ---- 632

RESEARCH ANNOTATION: THE CARIES-INHIBITING EFFECTS OF Na_2HPO_4 AND $CaHPO_4$. *Robert Van Reen and Kenneth F. Glassford*, Bethesda, Md. ---- 633

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH: Proceedings of the Northwest Branch ---- 634

No. 4, JULY—AUGUST, 1959

SUPPORTING ASSOCIATES FOR 1959 ---- 637

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. Announcement of 1960 General Meeting ---- 638

EDITORIAL VIEWS AND NEWS ---- 639

PRESIDENTIAL INAUGURAL ADDRESS. *Hamilton B. G. Robinson* ---- 640

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. Abstracts of papers presented at Thirty-Seventh General Meeting, San Francisco, Calif. March 21-22, 1959. *Compiled by Frank J. Orland* ---- 650

Abstracts of papers presented at General Meeting Sessions; Abstracts 1-98 650
Abstracts of papers presented at Symposiums I and II; Abstracts S1-S16 693
Abstracts of papers presented at General Meeting Sessions; Abstracts 99-220 700
Abstracts of papers presented to Dental Materials Group; Abstracts M1-M36 ---- 749
Index of Participants and Sequence Numbers for Abstracts ---- 763

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH: Proceedings of Thirty-Seventh General Meeting, March 19-22, 1959 ---- 766

FURTHER OBSERVATIONS ON THE CARIOSTATIC EFFECT OF PHOSPHATES. *F. J. McClure and A. Muller, Jr.*, Bethesda, Md. ---- 776

THE EVANSTON DENTAL CARIES STUDY. XIX. PREVALENCE OF MALOCCLUSION OF CHILDREN IN A FLUORIDATED AND CONTROL AREA. *I. N. Hill, J. R. Blayney, and W. Wolf*, Chicago, Ill. ---- 782

THE EFFECT OF ANTIHISTAMINICS ON DENTAL CARIES IN THE RAT. *Joseph C. Muhler and William G. Shafer*, Indianapolis, Ind. ---- 795

STUDIES ON THE ANTIBACTERIAL ACTION OF HUMAN SALIVA. I. A BACTERICIDIN FOR LACTOBACILLI, *Bernard J. Zeldow*, Seattle, Wash. ---- 798

THE EFFECTS OF VARIOUS LEVELS OF SWEET PEAS (LATHYRUS ODORATUS) IN THE DIET, WITH AND WITHOUT DIETARY SUPPLEMENTS, ON THE GROWTH OF THE MANIBULAR RAMUS AND ARTICULATION IN YOUNG RATS. *Alvin Frederick Gardner*, Washington, D. C. ---- 805

THE EFFECT OF OAT HULLS ON THE DENTAL CARIES EXPERIENCE IN RATS. *Wolfgang Buttner and Joseph C. Muhler*, Indianapolis, Ind. ---- 823

A "CLINICAL RULE": USED AS A MEASURING INDEX IN REPOSITIONING PERIODONTALLY-INVOLVED, MIGRATED TEETH. *Abraham Berliner*, New York City, N. Y. ---- 825

A NEW MOUTH PROP FOR ORAL EXAMINATIONS AND OPERATIVE PROCEDURES IN RODENTS. *A. A. Rizzo*, Bethesda, Md. ---- 830

THE CARCINOGEN 20-METHYLCHOLANTHRENE AND SALIVARY GLAND TRANSPLANTS. *Harold S. Fleming,* Washington, D. C. ---- 833

RESEARCH ANNOTATION: A NOTE ON THE OCCURRENCE OF ACTINOMYCES BOVIS IN THE HAMSTER. *Robert J. Gorlin, and Anand P. Chaudhry,* Minneapolis, Minn. ---- 842

RESEARCH ANNOTATION: WHOLE SALIVA VOLUME, SODIUM, POTASSIUM, AND CHLORIDE: DAY-TO-DAY COMPARISONS UNDER IDENTICAL AND DIVERSE FORMS OF STIMULATION. *Ira L. Shannon and John R. Prigmore,* Randolph AFB, Texas ---- 843

RESEARCH ANNOTATION: THE IMMUNOHISTOCHEMICAL LOCALIZATION OF PLASMA PROTEINS IN HUMAN GINGIVAE. *Jack D. Zwemer, Hsien-Ming Meng, and Robert L. Nutter,* Loma Linda, Calif. ---- 844

No. 5, SEPTEMBER—OCTOBER, 1959

SUPPORTING ASSOCIATES FOR 1959 ---- 845

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. Announcement of 1960 General Meeting ---- 846

EDITORIAL VIEWS AND NEWS ---- 847

THE EFFECTS OF SALIVA ON THE pH AND LACTATE CONCENTRATION IN DENTAL PLAQUES. I. CARIES-RAMPANT INDIVIDUALS. *Harold R. Englander, Irving L. Shklair, and Leonard S. Fosdick,* Chicago, Ill. ---- 848

ELECTROPHORESIS OF SALIVA. *J. H. Geller, L. Hames, and G. H. Rovelstad,* Bainbridge, Md. ---- 854

DIETARY HABITS AND THE DENTAL CARIES EXPERIENCE IN 200 CHILDREN. *Alegria C. Zita, Ralph E. McDonald, and Aaron L. Andrews,* Indianapolis, Ind. ---- 860

CORRELATION BETWEEN NERVOUS ACCOMMODATION, SYMPTOMATOLOGY, AND HISTOLOGY OF NORMAL AND PATHOLOGIC TOOTH PULP: ITS APPLICATION TO ELECTRODIAGNOSIS. *Fernando Vargas F., and Luz Vivaldi M,* Concepción, Chile ---- 866

THE EFFECT OF TOPICALLY APPLIED POTASSIUM FLUOROSTANNITE ON THE DENTAL CARIES EXPERIENCE IN CHILDREN. III. RESULTS AT THE END OF THREE YEARS. *Charles W. Gish, Joseph C. Muhler, and Charles L. Howell,* Indianapolis, Ind., and Bloomington, Ind. ---- 881

COMPARATIVE ANALYSIS OF SALIVA IN PREGNANT AND NONPREGNANT WOMEN. I. CALCIUM AND pH. *S. Leonard Rosenthal, Robert Rowen, and A. J. Vazakas,* Philadelphia, Pa. ---- 883

THE EFFECT OF HEAT TREATMENT VARIABLES ON THE MICROSTRUCTURE AND HARDNESS OF A CAST DENTAL GOLD ALLOY. *Anthony K. Kaires, and Joseph C. Thompson,* San Francisco, Calif., and San Diego, Calif. ---- 888

PRETREATMENT OF TEETH WITH SODIUM FLUORIDE PRIOR TO STANNOUS FLORIDE APPLICATION IN CHILDREN. *H. Abdul-Ghaffar and Joseph C. Muhler,* Indianapolis, Ind. 901

REDUCED ORAL TEMPERATURES AND ACID PRODUCTION RATES IN DENTAL PLAQUES. *William R. Stanmeyer and Robert J. Adams,* New London, Conn. ---- 905

SOLUTION RATE OF TOOTH ENAMEL IN AN ACETATE BUFFER, *R. S. Manly and Dorothy P. Harrington,* Westwood, Mass. ---- 910

METHODS OF INVESTIGATING THE VASCULAR ARCHITECTURE OF THE MANDIBLE. *Lawrence Cohen,* London, England ---- 920

SURFACE REPRODUCTION OF ELASTIC IMPRESSIONS. *Hiroyasu Hosoda and Takao Fusayama,* Tokyo, Japan ---- 932

FINE STRUCTURES OF THE DENTAL PULP. *James L. Matthews, Homer L. Dorman, and Jack G. Bishop,* Dallas, Texas ---- 940

THE IN VITRO EFFECTS OF ANTIBIOTICS ON ORAL SPIROCHETES. *E. G. Hampp and R. J. Fitzgerald,* Bethesda, Md. ---- 947

SALIVARY AND PLAQUE LACTOBACILLI IN THE PROGNOSIS OF HUMAN DENTAL CARIES. *Gordon E. Green and Paul R. Weisenstein,* Columbus, Ohio ---- 951

HISTOCHEMICAL LOCALIZATION OF HYDROLYTIC ENZYMES IN HUMAN SALIVARY GLANDS. *Howard H. Chauncey and Giuliano Quintarelli*, Boston, Mass. ---- 961

PERIPHERAL CAPILLARIES IN THE RODENT INCISOR PULP. *D. Adams*, Edinburgh, Scotland 969

STUDIES ON MUCOPOLYSACCHARASE ACTIVITY OF ORAL SPIROCHETES. *Edward G. Hampp, Stephan E. Mergenhagen, and Robert R. Omata*, Bethesda, Md. ---- 979

SURVIVAL OF MOUSE TOOTH GERMS AFTER FREEZING IN LIQUID NITROGEN AS DEMONSTRATED BY DIFFERENTIATION OF ISOLOGOUS TRANSPLANTS. *W. Yoshioka and F. Gonzales*, Houston, Texas ---- 983

THE COMBINED ANTICARIOGENIC EFFECT OF A SINGLE STANNOUS FLUORIDE SOLUTION AND THE UNSUPERVISED USE OF A STANNOUS FLUORIDE-CONTAINING DENTIFRICE. *Joseph C. Muhler*, Indianapolis, Ind.---- 994

THE AMINO ACID DECARBOXYLASE OF SALIVARY SEDIMENT. *N. Gochman, R. K. Meyer, R. Q. Blackwell, and L. S. Fosdick*, Chicago, Ill. ---- 998

CULTURE BROTH CONTAMINATION BY RESIDUAL PARA-AMINOTOLUENE SULFONAMIDE (BENZYLOG). *B. F. Gurney and E. James Best*, Chicago, Ill. ---- 1004

THE EFFECT OF HYPERVITAMINOSIS A UPON THE INCISOR AND MOLAR TEETH, THE ALVEOLAR BONE, AND TEMPOROMANDIBULAR JOINT OF WEANLING RATS. *Robert J. Gorlin and Anand P. Chaudhry*, Minneapolis, Minn. ---- 1008

AN ORAL FILAMENTOUS MICROORGANISM: CULTURAL CHARACTERISTICS AND MICROBIAL RELATIONSHIPS AFFECTING GROWTH. *Robert L. Richardson and Jean Schmidt*, Iowa City, Iowa ---- 1016

ADDITIONAL STUDIES ON THE SOLUBILITY OF CERTAIN DENTAL MATERIALS. *Richard D. Norman, Marjorie L. Swartz, and Ralph W. Phillips*, Indianapolis, Ind. ---- 1028

THE EFFECT OF CHRONIC VITAMIN A DEFICIENCY ON DENTAL CARIES IN THE SYRIAN HAMSTER. *John J. Salley, William F. Bryson, and J. Robert Eshleman*, Richmond, Va. ---- 1038

THE ORAL YEAST-LACTOBACILLUS RELATIONSHIP. II. YEAST-LACTOBACILLUS-SALIVA INTERRELATIONSHIPS. *Thomas E. Wilson and Paul W. Goaz*, Oklahoma City, Okla. 1044

RESEARCH ANNOTATION: THE EFFECT OF YEAST ON DENTAL CARIES IN THE RAT. *Wolfgang Buttner and Joseph C. Muhler*, Indianapolis, Ind. ---- 1052

RESEARCH ANNOTATION: THE EFFECT OF THE TOPICAL APPLICATION OF STANNOUS FLUORIDE ON ENAMEL SOLUBILITY WHEN PRETREATED WITH SODIUM FLUORIDE. *Joseph C. Muhler and Wolfgang Buttner*, Indianapolis, Ind. ---- 1053

RESEARCH ANNOTATION: ACID PRODUCTION AND THE CARIOUS PROCESS. *Alexander B. MacGregor*, Birmingham, England ---- 1055

RESEARCH ANNOTATION: COLLECTION OF MILLIGRAM QUANTITIES OF DENTAL ENAMEL DUST BY ELECTROSTATIC PRECIPITATION. *Leland C. Hendershot*, Midland, Mich. 1056

No. 6, NOVEMBER—DECEMBER, 1959

SUPPORTING ASSOCIATES FOR 1959 ---- 1057

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. Announcement of 1960 General Meeting ---- 1058

EDITORIAL VIEWS AND NEWS ---- 1059

ELECTROPHORESIS OF SALIVA; RELATIONSHIP OF PROTEIN COMPONENTS TO DENTAL CARIES. *J. H. Geller and G. H. Rovelstad*, Bainbridge, Md. ---- 1060

FRICTION AND PARTIAL DENTURE RETENTION. *J. A. Warr*, London, England ---- 1066

A COMPARISON OF THE DENTAL CARIES EXPERIENCE IN MALE AND FEMALE RATS RECEIVING THE SAME AMOUNT OF CARIOGENIC DIET. *Joseph C. Muhler*, Indianapolis, Ind. 1075

THE RELATIONSHIP BETWEEN FLUORIDE INGESTION AND URINARY CALCIUM. *Martin J. Wagner and Joseph C. Muhler*, Indianapolis, Ind. ---- 1078

HISTOCHEMISTRY OF BONE MATRIX DURING REST AND RESORPTION. *James A. Yaeger*, Chicago, Ill. ---- 1082

INVESTIGATIONS INTO THE MINERALIZATION PATTERN OF HUMAN DENTAL ENAMEL. *J. H. Allan*, Sheffield, England.
I. Polarized Light Studies ---- 1096
II. X-ray Absorption Studies ---- 1108
III. Decalcified Section Studies ---- 1119

A COMPARISON BETWEEN THE EFFECT OF SELECTIVE SALIVARIADENECTOMY AND SELECTIVE SALIVARY GLAND LIGATION ON DENTAL CARIES IN THE RAT. *Joseph C. Muhler, William G. Shafer, and David Bixler*, Indianapolis, Ind. ---- 1129

ELECTRON MICROSCOPIC STUDIES ON NERVES OF HUMAN TOOTH PULP. *Koji Uchizono and Kuninori Homma*, Niigata, Japan ---- 1133

EFFECT OF FOOD ASH, PHOSPHATE, AND TRACE MINERALS UPON HAMSTER CARIES. *Robert S. Harris and Abraham E. Nizel*, Cambridge, Mass. ---- 1142

AIR PRESSURE DEVICE FOR THICKNESS MEASUREMENT OF UNDECALCIFIED TISSUE SECTIONS. *Barrie Gillings, Michael Buonocore, and Paul Pagerey*, Rochester, N. Y. 1148

AN APPARATUS FOR THE PREPARATION OF THIN SERIAL SECTIONS OF UNDECALCIFIED TISSUES. *Barrie Gillings and Michael Buonocore*, Rochester, N. Y. ---- 1156

STUDIES ON SALIVARIADENECTOMIZED HUNT-HOPPERT CARIES-RESISTANT AND OSBORNE-MENDEL-SUSCEPTIBLE (OMS) RATS. IV. EFFECT OF SELECTIVE SALIVARIADENECTOMY ON DENTAL CARIES AND SEVERAL GROUPS OF THE ORAL MICROFLORA. *S. Rosen, L. M. Sreebny, C. A. Hoppert, H. R. Hunt, and E. Bachem*, Chicago, Ill. 1166

NUTRITION AND CARIES. IX. THE INFLUENCE OF CALCIUM CARBONATE ON RAT CARIES INDUCED BY A CARIOGENIC DIET. *L. M. Dalderup*, Amsterdam, The Netherlands 1173

LACK OF SMOOTH-SURFACE CARIES IN RATS RECEIVING MODIFIED STEPHAN DIET. *Joseph C. Muhler*, Indianapolis, Ind. ---- 1178

MICRORADIOGRAPHIC AND POLARIZED LIGHT STUDIES OF INITIAL CARIOUS LESIONS. *Narendar Nath Soni and Finn Brudevold*, Boston, Mass. ---- 1187

SOME AGE CHANGES IN THE PERIODONTIUM OF THE ALBINO MOUSE. *Neville D. Gilmore and Irving Glickman*, Boston, Mass. ---- 1195

THE EFFECT OF EDTA ON THE PATTERN OF CARIES DEVELOPMENT AND ITS ASSOCIATION WITH BIOLOGIC CHANGES IN THE RAT. *Rachel H. Larson*, Bethesda, Md. ---- 1207

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH. BRITISH DIVISION. Abstracts of papers presented at Seventh Annual Meeting, April 9 and 10, 1959. *Compiled by I. R. H. Kramer* ---- 1213
Index of Participants and Sequence Number for Abstracts ---- 1234

RESEARCH ANNOTATION: FOOD CONSUMPTION AND DENTAL CARIES IN SALIVARIADENECTOMIZED AND DUCT-LIGATED RATS. *David Bixler, Joseph C. Muhler, and William G. Shafer*, Indianapolis, Ind. ---- 1235

RESEARCH ANNOTATION: EFFECT OF STANNOUS FLUORIDE DENTIFRICE FOLLOWING TOPICAL STANNOUS FLUORIDE APPLICATION ON DENTAL CARIES AND ENAMEL SOLUBILITY IN THE RAT. *Joseph C. Muhler*, Indianapolis, Ind. ---- 1236

RESEARCH ANNOTATION: PAROTID SALIVA SERUM AND URINE 17-HYDROXYCORTICOSTEROIDS FOLLOWING A TWO-HOUR INTRAVENOUS INFUSION OF ADRENOCORTICOTROPIN. *Ira L. Shannon, John R. Prigmore, Robert A. Brooks, and Ralph P. Feller*, Brooks Air Force Base, Texas ---- 1237

RESEARCH ANNOTATION: THE OCCURRENCE OF PERIODONTAL DISEASE IN GERMFREE MICE. *Paul N. Baer and Walter L. Newton*, Bethesda, Md. ---- 1238

RESEARCH ANNOTATION: THE IMMUNOHISTOCHEMICAL LOCALIZATION OF PLASMA ALBUMIN IN MURINE DENTINAL TISSUE. *Jack D. Zwemer and Ralph R. Steinman, With the Technical Assistance of C. James Heinrich*, Loma Linda, Calif. ---- 1239

RESEARCH ANNOTATION: CARIES POTENTIATING EFFECT OF Na EDTA, Ca EDTA, AND Mg EDTA IN THE RAT. *I. Zipkin and R. H. Larson*, Bethesda, Md. ---- 1240

INDEX ---- 1241

Vol. 38 No. 1

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Editorial News and Views ---------- 1

International Association for Dental Research. Announcement of 1959 General Meeting ---------- 2

Nitrogen Content of Zones Distinguished by Reflected Light in Enamel Sections From Unerupted Third Molars. L. W. Wachtel, Lieutenant Commander, MSC, USNR, Bethesda, Md. ---------- 3

Scatter Radiation Emanating From the Patient During Dental Roentgenographic Procedures. Duane W. Lovett, Iowa City, Iowa ---------- 9

A Comparative Histologic Study of Human Periodontal Structures Utilizing Fluorescence and Visible Light Microscopy. Alvin C. Hileman, Columbus, Ohio 15

The Effects of Beta-Aminopropionitrile Upon the Molar Teeth of the Rat. George A. Krikos, Rochester, N. Y. ---------- 27

Radiation Output of Dental X-ray Units. Duane W. Lovett, Iowa City, Iowa ---------- 36

(*Contents continued on inside back cover*)

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

THE JOURNAL OF DENTAL RESEARCH

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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

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Objects (Const., Art. II): The association has been established to promote broadly the advancement of research in all branches of dental science and in the related phases of the sciences that contribute directly to the development of oral health service, and as a whole; and further, to encourage and facilitate cooperative effort and achievement by, and mutual helpfulness among, investigators in all nations in every division of stomatology to the end that dentistry may render cumulatively more perfect service to humanity.

Membership: Total (October, 1958), 938. *Eligibility (Const., Art. III, Sec. 1).*—"Any person who has conducted, and published an account of, a meritorious original investigation in dental science, or in any of the sciences contributory to oral health, shall be eligible to membership in this Association, providing such person conforms to the recognized standards of professional ethics." *Election.*—New members may be elected *only at general meetings of the Association.* Membership in a section is secondary to membership in the Association as a whole. Removal of members from one geographic center to another does not affect their membership in the Association.

Nomination blanks for membership in this Association may be obtained on request from the Secretary-Treasurer.

Endowment fund of the International Association for Dental Research (as of October, 1958): $1,891.01. There have been no expenditures from this fund.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
37th General Meeting
Sheraton-Palace, San Francisco, California
March 19-22, 1959
For further instructions, contact Dr. Burrill, Sec.-Treas.

THE JOURNAL OF DENTAL RESEARCH

This JOURNAL is an official publication of the International Association for Dental Research. It is dedicated to the dissemination of knowledge pertaining to the oral cavity, its contents and contiguous parts, and its relation to and effect on the total human organism. The JOURNAL publishes original investigations in these and related fields. Articles are accepted only if they are contributed solely to this JOURNAL.

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All manuscripts and correspondence should be addressed to the Editor: Dr. F. J. Orland, Zoller Dental Clinic, University of Chicago, 950 E. 59th Street, Chicago 37, Illinois.

EDITORIAL VIEWS AND NEWS

Ordinarily, the Editor would prefer to look ahead to Volume 38 of the JOURNAL rather than to reminisce about the several Numbers published in 1958. Nevertheless, a few interesting elements of publication might be cited: In the six issues comprising Volume 37, there were a total of 122 manuscripts published. This is a significant increment over the 71 manuscripts published in 1948 (805 pages), the 47 in 1938 (532 pages), and the meager 16 in 1928 (550 pages). There has hardly been a shortage of submitted manuscripts in recent years, but the greater number of pages (1186 arabic numbered, plus 22 more concerned with indices and comments), in the 1958 JOURNAL, which permitted the publication of more manuscripts and additional features, was made possible largely by the grant of the William J. Gies Foundation announced at the Detroit I. A. D. R. meeting.

To publish a scholarly journal these days costs a substantial sum, even though the very significant services of the Advisory Editorial Board and the several Editors are all donated to the cause. It is a comforting thought that such worthy groups as the Gies Foundation, the National Institute of Dental Research, through its extramural grants program, and the Supporting Associates of the I. A. D. R. —*J. D. Res.* fully appreciate this fact and are assisting in the publication cost problem of this JOURNAL.

Use of Professional and Academic Degrees. —Beginning with this January—February issue of the JOURNAL, academic and professional degrees will no longer appear after the authors' names. This action is in keeping with the custom of all the better research journals; moreover, the listing of degrees should not be necessary as a mark of the author's competence. In fact, many contributors to the *J. D. Res.* have chosen voluntarily not to include their degrees for some time now. After all, it is not the exhibition of hard-earned degrees that is as important as the content of the manuscript *per se*.

A Biographical Directory.—On the other hand, to provide academic and professional background information, there should be some reliable source book wherein a brief, biographical sketch is available concerning persons active in oral research. *American Men of Science* is an excellent general compendium for this kind of information but, unfortunately, it does not include the names of the younger and less-established investigators, nor of those from across the seas. Thus, the Editor strongly suggests the time has come for the I. A. D. R. to consider the preparation of another Biographical Directory such as Harold Hodge, Ed Hatten, and H. B. G. Robinson prepared in the JOURNAL of October, 1952. The present Editor would recommend, however, that biographical sketches be included only for those I. A. D. R. members not already listed in the most recent volume of *A.M.S.* These latter individuals should be listed merely by name (with some appropriate symbol) and current address. In the questionnaire to I. A. D. R. members regarding the JOURNAL, it was suggested by some that this formidable task might be best accomplished by the Archives Committee of the International Association for Dental Research.

Constructive Comments.—Comments to the Editor regarding the JOURNAL are always welcome. (How else would we learn of such almost humorous incidents as the ironic case of F. A. Arnold, of all people, and his copy of the last issue of the JOURNAL with the blank pages?) Constructive comments are especially welcome at this time while the Editor's Annual Report is being prepared for the next I. A. D. R. meeting.

—FRANK J. ORLAND
EDITOR

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
ANNOUNCEMENT OF 1959 GENERAL MEETING

The 37th General Meeting of the International Association for Dental Research will convene at the Sheraton-Palace, San Francisco, California, on Thursday, Friday, Saturday, and Sunday, March 19, 20, 21, and 22, 1959. The Dental Materials Group of the Association will meet on Friday, Saturday, and Sunday, March 20, 21, and 22. The American Association of Dental Schools will meet at the same place beginning on Sunday, March 22.

DAN Y. BURRILL, SECRETARY-TREASURER
Northwestern University Dental School
311 E. Chicago Avenue
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NITROGEN CONTENT OF ZONES DISTINGUISHED BY REFLECTED LIGHT IN ENAMEL SECTIONS FROM UNERUPTED THIRD MOLARS

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U. S. Naval Dental School, National Naval Medical Center, Bethesda, Md.

NUMEROUS investigators[1-6] have reported varying amounts of protein and nitrogen, ranging from about 0.4 per cent protein (0.07 per cent nitrogen) to about 2.0 per cent (0.3 per cent nitrogen), in normal, noncarious enamel. Interest in this area of dental research has continued in an effort to ascertain a relationship between the organic content of enamel and the susceptibility of teeth to caries.

In almost all work done in the past, samples used for analysis were pooled from fairly large numbers of teeth, and the purity of the enamel was established by means of density determinations. These procedures, however, did not establish individual differences, and may have excluded nitrogen-rich areas of enamel having a lower density than normal enamel.

In 1951, Coolidge[7] attempted to correct these faults by comparing small samples from the same tooth. Although at that time he could establish no consistent relationship between normal enamel, carious enamel, and hard white spots of enamel, he has since questioned the reliability of his sampling procedures. Stack,[8] using an improved sampling technic, was not able to detect any variation in the organic content of sound enamel at various points around the crown, but did observe that chalky enamel contained 3 to 4 times as much organic matter as the adjacent areas.

The present studies were initiated in an effort to obtain more precise information on the distribution of nitrogenous matter in enamel. The use of undemineralized tooth slices permitted the removal and analysis of areas observed in the inner regions of the enamel. This type of sampling allowed a more careful selectivity than that obtained with flotation technics, and permitted the determination of individual variations. Since the samples used were small, it was necessary to adopt an ultramicro-Kjeldahl technic to measure the nitrogen content.

MATERIALS AND METHODS

Samples.—Samples were obtained from unerupted third molars that had been stored in a freezer immediately after extraction. The effects of oral

Preliminary results of this study were reported at the 35th General Meeting of the International Association for Dental Research, Atlantic City, March 21, 1957.

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The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

environment and age were minimized by confining samples to teeth obtained from 18- to 20-year-old persons. The teeth were cleaned and brushed thoroughly and then cut buccolingually, by means of a Brown and Sharpe 0.01-HS-S circular saw, into sections about 200 μ thick. Each slice was examined microscopically as a further check against caries that may have resulted from an undisclosed oral communication. Under reflected light, areas of enamel appeared clear, cloudy white, or opaque yellow (Fig. 1). Selected areas suitable for analysis were removed by fracturing with a Zerfing scaler, examined microscopically, air dried, and weighed.

Nitrogen Determinations.—The ultramicro-Kjeldahl technic devised by Needham and Boell[9] was the basic procedure followed. Samples were placed in small digestion flasks (Fig. 2, *a*) along with a few milligrams of selenium powder and 0.15 ml. of a digestion solution made up of equal parts of concentrated sulfuric acid and saturated potassium sulfate solution. The samples were then digested overnight in an electric sand bath heated to 250° C. After digestion, water was added, and the solution was made alkaline by adding several sodium hydroxide pellets. The released ammonia was allowed to diffuse overnight into the waxed receptor tubes (Fig. 2, *b*) containing 0.1 ml. of 0.03 N sulfuric acid. Diffusion was aided by placing the tubes on a rocking device in a water bath heated to 38° C. Analysis of the receptor tube contents was made colorimetrically by the Russell alkaline-phenol-hypochlorite procedure.[10]

Phosphorus Determinations.—After the material remaining in the digestion flask had been adjusted to pH 2, the phosphorus content was measured by the standard Fiske-Subbarow method.[11]

Phosphorus-Nitrogen Ratio.—Both phosphorus and nitrogen determinations were made on the same samples. The phosphorus-nitrogen ratio was taken to indicate the relative proportions of inorganic and organic components.

TABLE I
RECOVERIES OF NITROGEN AND PHOSPHORUS FROM CALCIUM PHOSPHATE—KERATIN MIXTURES BY MICRO AND ULTRAMICRO TECHNICS

$Ca_3(PO_4)_2$: KERATIN RATIO	WEIGHT OF ULTRAMICRO SAMPLES (MG.)	PHOSPHORUS (PER CENT)		NITROGEN (PER CENT)		P/N	
		MICRO*	ULTRAMICRO†	MICRO*	ULTRAMICRO†	MICRO*	ULTRAMICRO†
100:1	0.7 to 3.9	17.4	17.0 ± 0.8‡	0.11	0.15 ± 0.04	158	125 ± 32
20:1	0.4 to 2.3	16.7	16.5 ± 1.2	0.55	0.58 ± 0.11	30	30 ± 6
20:1	0.05 to 0.3					30	27 ± 8

*Weight of samples = 50—300 mg.
†10—20 samples/mixture.
‡Standard deviation.

THE JOURNAL OF DENTAL RESEARCH
JANUARY—FEBRUARY, 1959

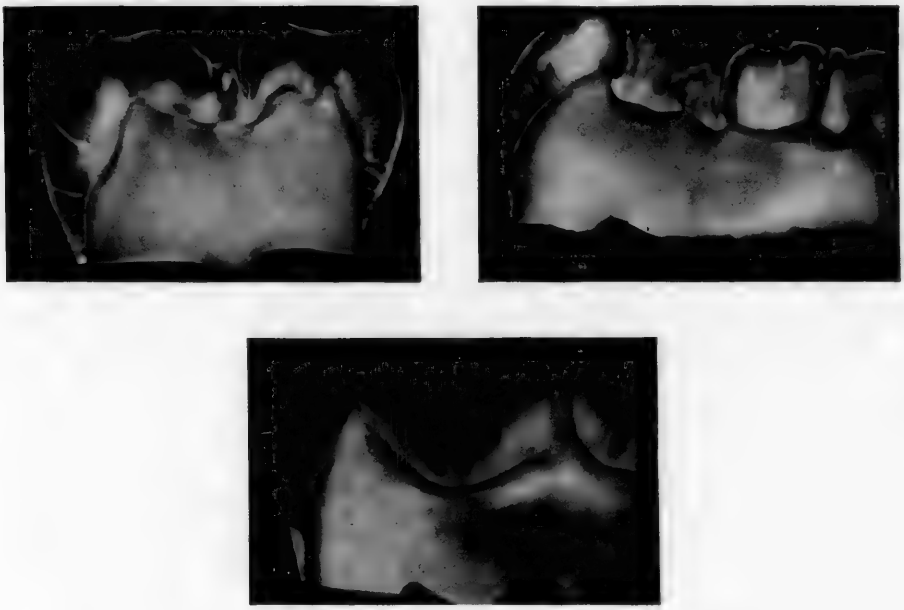

Fig. 1.—Tooth slices showing clear, cloudy white, and opaque yellow areas of enamel.

Accuracy of Methods.—The degree of accuracy of the procedures was established, using mixtures of calcium phosphate and keratin, by comparing ultramicro results with those obtained by standard micro procedures. Table I shows the results of this comparison. A coefficient of variation (σ/mean) of about ±20 per cent was obtained with samples weighing from 0.4 to 3.9 mg. and containing from 1 to 10 μg of nitrogen.

Although they did not affect the phosphorus-nitrogen ratios, weighing errors reduced the reliability of percentage values for samples less than 0.4 mg. Consequently, percentages were reported only for quantities exceeding that amount. As a further precaution, sample sizes were selected for reporting which contained at least 0.8 μg of nitrogen. The presence of a constant blank of 0.6 μg of nitrogen (introduced from the digestion materials) made it necessary to deduct that amount from all recoveries.

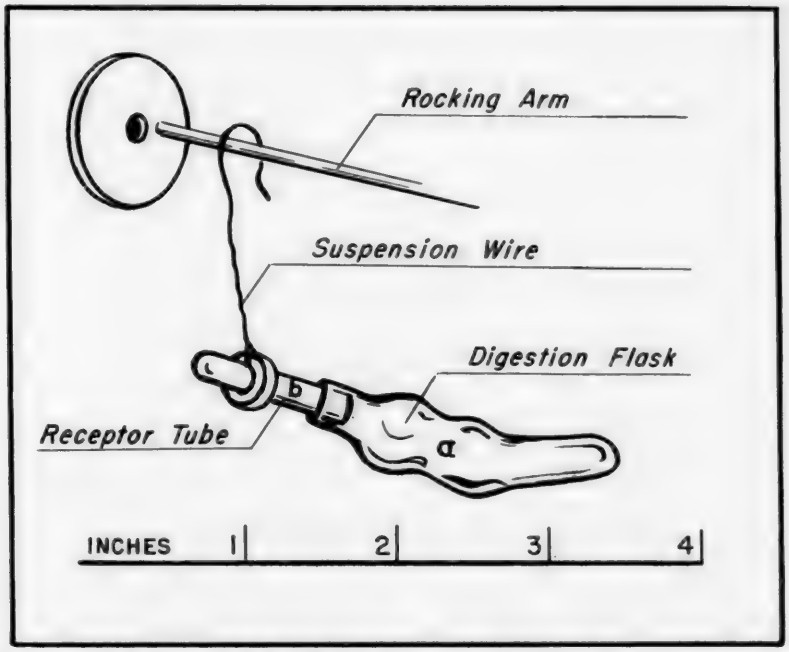

Fig. 2.—Diagram of digestion flask (*a*) and receptor tube (*b*) used in determination of nitrogen and phosphorus.

RESULTS

The results of analyses of tooth fragments of clear enamel, cloudy white enamel, opaque yellow enamel, and dentin are listed in Table II. Twenty teeth from 12 persons were used to compile these data. On the average, cloudy white enamel contained about 2½ times as much nitrogen as clear enamel, and had a phosphorus-nitrogen ratio of 159:1, compared with 358:1 for clear enamel. Opaque yellow enamel contained 6 times as much nitrogen as clear enamel; this nitrogen content associated with a decreased phosphorus content

gave opaque yellow enamel a phosphorus-nitrogen ratio of 41:1. The comparative differences in nitrogen content and phosphorus-nitrogen ratio between the various types of enamel are statistically significant ($P<0.01$).

TABLE II
ANALYSES OF ENAMEL TYPES FOR NITROGEN AND PHOSPHORUS

TYPE	SAMPLES (NO.)	WEIGHT OF SAMPLES (MG.)	PHOSPHORUS (PER CENT)	NITROGEN (PER CENT)	P/N
Clear enamel	63	0.8 to 7.3	17.7 ± 1.6*	0.07 ± 0.04	358 ± 213
Cloudy white enamel	41	0.4 to 3.3	17.1 ± 1.2	0.17 ± 0.11†	159 ± 127†
Opaque yellow enamel	17	0.4 to 1.9	16.4 ± 1.8‡	0.43 ± 0.11§	41 ± 12§
Dentin	33	0.4 to 1.4	12.3 ± 1.6	2.46 ± 0.40	5.1 ± 1.1

*Standard deviation.
†Clear enamel vs. cloudy enamel $P<0.01$.
‡Clear enamel vs. opaque enamel $P<0.01$
§Cloudy enamel vs. opaque enamel $P<0.01$.

In order to compare the nitrogen content of different areas of enamel within the same tooth, the results of analyses of 16 teeth were compiled (Table III). These values indicate that the relative amounts of nitrogen in the 3 areas vary greatly in different tooth samples, and the differences are more striking than in Table II; the mean nitrogen content of cloudy white enamel was 3.2 times greater, and that of opaque yellow enamel 10.2 times greater, than the mean nitrogen content of clear enamel from the same tooth.

TABLE III
COMPARATIVE RATIOS OF ENAMEL TYPES FROM INDIVIDUAL TEETH, BASED ON NITROGEN CONTENT

TOOTH NO.	CLEAR (E) (PER CENT N)	CLOUDY (E′) (PER CENT N)	OPAQUE (E″) (PER CENT N)	COMPARISON E′/E	COMPARISON E″/E
1	0.11	0.18	†	1.6	
2	0.07	0.12	0.50	1.7	7.1
3	0.08	0.22	†	2.8	
4	0.04	†	0.20		5.0
5	0.04	0.02	0.46	0.5	11.5
6	0.02	†	0.42		21.0
7	0.03	0.20	0.48	6.7	16.0
8	0.08	0.13	†	1.6	
9	0.06	0.17	†	2.8	
10	0.10	0.36	†	3.6	
11	0.07	0.32	†	4.6	
12	0.06	0.15	0.41	2.5	6.8
13	0.06	0.25	†	4.2	
14	0.05	0.33	0.42	6.6	8.4
15	0.04	0.14	†	3.5	
16	0.07	0.13	0.43	1.9	6.1
Average				3.2 ± 1.8‡	10.2 ± 5.6

†Not present.
‡Standard deviation.

The phosphorus-nitrogen ratios, included in the results, are extremely useful where percentages may be unreliable because of sample size. It should be noted that the ratios listed in the last column of Table II were obtained

by averaging all individual determinations in each group. They differ slightly from ratios calculated from the average percentages of phosphorus and nitrogen shown in the same table.

Despite difficulties, the procedures used gave results with relatively small standard deviations (Table I). The values shown in Table II conform with those reported previously for a smaller number of samples.[12]

The nitrogen and phosphorus values are within the general range obtained by other investigators studying the composition of enamel. The careful separation of opaque yellow enamel from clear enamel, however, exposed differences in composition which existed between these morphologically different areas. The observation of these significant differences between enamel areas of the same tooth stresses the value of visual sampling, rather than the use of pooled samples, in determining variations in individual teeth.

The nitrogen content of the opaque yellow enamel, although relatively high when compared with the nitrogen content of clear enamel, is relatively low when compared with the nitrogen content of dentin. No individual sample of enamel contained more than 0.7 per cent nitrogen. This obervation together with the diffuse pattern of opacity noted on stereoscopic examination emphasizes the dispersed nature of nitrogen in enamel.

If the nitrogen content may be assumed to indicate the amount of protein present (obtained by multiplying the amount of nitrogen present by the factor 6.25*), the areas richest in nitrogen detected in the enamel contained about 3 to 4 per cent in protein. As they are often observed at the base of natural fissures, it may be that these nitrogen-rich areas serve as an entry point for invading organisms. Future efforts, therefore, will be directed toward determining their relation to susceptibility to bacterial and acid attack.

SUMMARY

1. Undemineralized tooth slices, 200 μ thick, were obtained from unerupted third molars of young adults, and fragments of enamel were sectioned according to their opacity to reflected light.

2. Selected samples weighing from 0.4 to 7.3 mg. were analyzed for phosphorus and nitrogen. (A modified ultramicro-Kjeldahl technic was adapted for the nitrogen determinations.)

3. The average nitrogen content of clear enamel for all teeth was 0.07 per cent; of cloudy white enamel 0.17 per cent; and of opaque yellow enamel 0.43 per cent. (Air-dried dentin contained about 2.5 per cent nitrogen.)

4. Comparison of the nitrogen content and of the phosphorus-nitrogen ratios of different areas of enamel from the *same* tooth revealed that, on the average, cloudy white enamel contained about 3 times as much nitrogen, and opaque yellow enamel about 10 times as much nitrogen, as clear enamel.

I wish to acknowledge with thanks the skilled technical assistance of Mr. Irving J. Henry and Mr. John W. Hathaway, and the helpful advice of Captain M. G. Wheatcroft, DC, USN.

*Stack[6] has reported that enamel keratin contains 13 per cent nitrogen, indicating that the conversion factor may be 7.75.

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SCATTER RADIATION EMANATING FROM THE PATIENT DURING DENTAL ROENTGENOGRAPHIC PROCEDURES

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THE amount of scatter radiation emanating from the patient during dental roentgenographic procedures is a necessary factor in attempting to determine the adequate protective measures for safety of those performing such procedures. This investigation was performed for the purpose of determining, by gross radiation detection equipment, the scatter radiation emanating from the patient during routine dental roentgenographic procedures in a specific dental facility.

PROCEDURE

X-Ray Equipment.—The investigation involved the use of four different dental x-ray units.* Each of these dental x-ray units was installed in almost identical cubicles, approximately 7 by 10 feet in size, with leaded walls on three sides. Each cubicle contained an x-ray unit, a dental chair, a film dispenser, and film receptacle mounted on the side walls, 4½ feet from the floor. The x-ray units were positioned at the center of the back wall of the cubicle with the dental chair positioned directly in front of the x-ray unit. All of the equipment used in this phase was in excellent operating condition and unaltered in any way except in that part of this study in which additional primary beam aluminum filters, which is standard with long cone equipment, were utilized. Each unit was operated according to the manufacturer's recommendations. A specific caution was exercised to have the timing device of each unit adjusted to its finest degree of accuracy.

X-Radiation Detection Equipment.—This consisted of several kinds of detection apparatus.†

The Dosometers were positioned by the use of an adjustable pedestal type stand as shown in Fig. 1. The stand supported the Dosometer by a plastic bracket completely backed by lead sheeting, 1/16 inch in thickness, as shown in Fig. 2. The mobility of the lightweight stand and the free floor space around the dental chair gave excellent access for positioning the Dosometers at any desired distance, height, and angle in respect to the patient in the dental chair.

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*A General Electric, CDX model E; a Fisher, Model MD No. M-1135; a Weber Radex, Model No. 11; and a Ritter Dual X, Model D.

†Two Model K-112 Keleket Dosometers with a 0 to 200 mr range, a Dosometer Charging Unit, Radiac Detector Charger (PP-630-PD), a Radiation Monitor by General Electric (Model 4SN11A3), Keleket Film Badges (Model K-180), a Photovolt electronic transmission densitometer (Models 52 and 501A), and a Kodak densitometer (Model 1).

Preliminary Experiments and General Procedure.—The establishment of the procedure in measuring the scatter x-radiation at specific distances from the patient's head during x-radiation exposure required the examination of several factors that might contaminate or alter the actual measurement of the scatter x-radiation specifically from the patient. The head of each of the

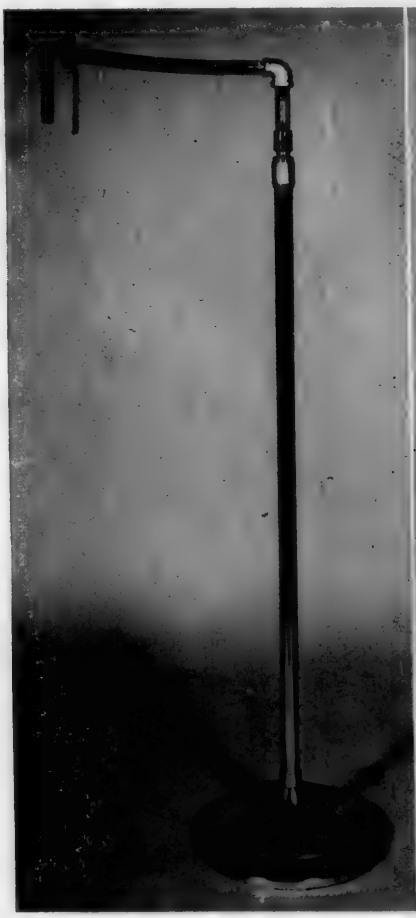

Fig. 1.

Fig. 2.

Fig. 1.—Adjustable stand used in supporting and positioning of Dosometer.
Fig. 2.—The supporting and shielding apparatus for the Dosometer.

x-ray units used in this study was meticulously examined for radiation leakage, outside of the area of the outlet of the primary beam. This examination was performed by completely covering the tube ahead except for one inch from the cone area with unexposed dental films, retained in position by cellophane tape. Each of the x-ray unit heads covered in this manner were energized for a total of 150 seconds by intermittent periods of 5 seconds with

the primary beam directed horizontally toward a leaded wall, at a distance of 5 feet. The exposure procedure required approximately 30 minutes to perform. All films were processed in the same solutions at the same time for each x-ray unit being examined. Naked eye examinations of the processed films with the use of diffused fluorescent lights disclosed no x-radiation effect on the films. The films were examined by using first one layer of frosted glass over the light source; and third, by three layers of frosted glass over the light source. This type of examination was performed to determine whether the alteration of the density of the fluorescent light source might aid in visual film examination. However, the direct visual examination was negative. The test films were each examined by use of the electronic transmission densitometer. This manner of film examination did reveal some variances of film densities but were judged to be so slight that the difference could also be confused with normal film variances. This conclusion was based on past experience in testing dental x-ray film, and not by experimentation within this study.

The Keleket Dosometers were to be used in the detection of scatter x-radiation as well as x-ray film, and in order to eliminate any question as to the sensitivity of either method of x-radiation, each of the x-ray tube heads was examined by a similar procedure with the Dosometer. With the use of three Dosometers, with a scale range of 0 to 200 mr, two were placed on one side of the tube head at a time, retained by cellophane tape, and the unit energized for a total of 100 seconds by intermittent periods of 5 seconds. Each of the 6 sides of the tube head was similarly examined and each of the 4 x-ray units examined in the same manner. The third Dosometer was used as a control—the control was placed in a lead-lined container and maintained as near as possible the same temperature as the testing Dosometers on the tube heads. The control Dosometer only gave an indication of the drift rate of the meters during the experimentation period. The reading of the experimental Dosometers and the control Dosometer was made immediately following the completion of the examination of each side of the tube head, which would be after 100 seconds of tube usage. In each case, the reading on the experimentation Dosometer was practically identical to the reading of the control Dosometer. With this information, the possibility of scatter x-radiation from the tube head would not be considered to be a contaminant in the determination of the scatter x-radiation emanating from the patient.

The investigation of scatter x-radiation emanating from the walls of the cubicle was not undertaken in this study since this subject has been investigated at the National Bureau of Standards by Scott W. Smith.[1] During the experimental procedures, the position of the Dosometers was never closer than 3 feet to any wall. Without actual determination of scatter x-radiation from the leaded walls, this factor was not considered as a contaminant factor. This interpretation may be in error but, since funds and adequate equipment were not available for investigating this phase of the problem, the interpretation was accepted by the investigator.

The investigation of scatter x-radiation from the dental chair and other parts of the x-ray unit other than the tube head was not accomplished because none of the equipment came in direct contact with the primary beam of radiation before it passed through the patient, except for the chair headrest. The metal support of the headrest was in the primary x-radiation beam in some exposures of lower posterior teeth when using the 8 or 9 inch film-target distance technics. However, the Dosometer was always positioned in such a manner as to have the patient's head between it and the exposed headrest, thus tending to eliminate any direct contamination from that source.

Fig. 3.—The Dosometer positioned for x-radiation detection during dental roentgenographic procedures.

The clinical determination of scatter x-radiation emanating from the exposure of the patient to the direct beam of x-radiation was routinely and precisely performed in the same manner regardless of the x-ray unit being used. Four dental x-ray units used in this study will be designated as Unit A, Unit B, Unit C, and Unit D.

Clinical Procedure.—The roentgenographic room was prepared for the patient's reception by x-ray unit testing and adjusting and preparation of the Dosometer and its placement in the adjustable holder. The patient was instructed as to the procedure being performed and prepared for the x-radiation exposure as shown in Fig. 3. When the film and the x-ray tube head had

been properly positioned and adjusted, immediately prior to the x-radiation exposure, the Dosometer was located in a position 4 feet from the floor, at a right angle to the direction of the direct beam of primary x-radiation and at a specified distance from the face of the patient. The x-radiation exposure was made and the procedure duplicated for each subsequent exposure for each patient during that clinical period. During the time the Dosometer was not in actual use, and between patients, it was retained in a lead-lined x-radiation safe receptacle. Most of the readings were made on patients receiving a full-mouth roentgenographic survey, and Dosometer readings were made following its use with each patient, as well as at the end of a clinical period which was approximately 2½ hours. No attempt was made to introduce corrections for temperature, humidity, and meter drift. The drift factor was not calculated because the longest possible period between readings was less than 2½ hours.

RESULTS

Two specific dental roentgenographic technics were used in this study and they will be designated as the short film-target distance technic and the long film-target distance technic. The short film-target distance technic will designate a skin-target distance of approximately 8 inches. The long film-target distance technic will designate a skin-target distance of 17 inches.

The area of coverage by the primary x-radiation at the patient tissue surface, using the short skin-target distance was a circle 4½ inches in diameter of 15.90 square inches. The area of coverage by the primary x-radiation at the tissue surface using the long skin-target distance was a circle 3 inches in diameter or 7.07 square inches. The tabulation of the findings using the short film-target distance are shown in Table I, the findings using the long film-target distance are shown in Table II. The tissue Dosometer distance

TABLE I
SHORT FILM-TARGET DISTANCE

X-RAY UNIT	TISSUE DOSOMETER DISTANCE (INCHES)	TOTAL TIME (INTERMITTENT EXPOSURE, SEC.)	TOTAL DOSOMETER READING (MR)	AVERAGE MR PER 4 SEC. EXPOSURE
C	24	1,240	142	0.46
A	24	1,080	120	0.44
A	30	1,325	147	0.35
B	30	1,440	176	0.49

TABLE II
LONG FILM-TARGET DISTANCE

X-RAY UNIT	TISSUE DOSOMETER DISTANCE (INCHES)	TOTAL TIME (INTERMITTENT EXPOSURE, SEC.)	TOTAL DOSOMETER READING (MR)	AVERAGE MR PER 4 SEC. EXPOSURE
A	24	1,371	82	0.24
A	30	1,382	50	0.17
B	30	1,200	28	0.09
D	54	2,314	28	0.048
D	72	2,457	16	0.026

indicates the distance between the Dosometer and the skin during x-radiation exposures. The total time designates the total number of seconds of intermittent exposures made and the total Dosometer reading is the amount of x-radiation detected. The average milliroentgen per exposure was calculated by using an arbitrary exposure time of 4 seconds.

The scatter x-radiation detected at the 24-inch distance from the tissue, under the same conditions, varied with the two roentgenographic technics used in this study. The short film-target distance technic with the target-skin distance of 8 inches gives a calculated reading of 0.46 mr and 0.44 mr per 4-second exposure. The long film-target distance technic with a target-skin distance of 17 inches gives a calculated reading of 0.24 mr per 4-second exposure. The scatter x-radiation detected at the 30-inch distance from the tissue also varied with the two roentgenographic technics used. The short film-target distance technic with the target-skin distance of 8 inches gives a calculated reading of 0.35 mr and 0.49 mr per exposure. The long film-target distance with a target-skin distance of 17 inches gave calculated readings of 0.17 mr and 0.09 mr per exposure. However, the readings of Unit B in Table I and Table II do not coincide with the readings obtained with the other units. This wide discrepancy would indicate errors in the use of this unit. The recordings in the 54-inch and 72-inch distances only indicate the decrease in detection of scatter x-radiation as the distance between the source of the scatter x-radiation and the detection instrument is increased. The determination of the quality of the scatter x-radiation recorded by this study was not undertaken, so that a correlation between the x-radiation detection findings in this study and total body x-radiation from scatter x-radiation cannot be made.

SUMMARY OF FINDINGS

The measurement of scatter x-radiation emanating from the face of a patient during dental roentgenographic procedures by gross x-radiation detection equipment shows a significant amount of x-radiation activity within 30 inches of the area being irradiated. The decrease in detected x-radiation with the greater film-target distance and smaller irradiated area is sufficient to indicate that this type of technic is very advantageous in regard to scatter x-radiation. The variation in the x-radiation detected with the use of different x-ray units under the same conditions indicates that a study of the x-radiation output of dental x-ray equipment is indicated. Scatter x-radiation present during dental roentgenographic procedures is at a sufficiently high level to require protective measures by and for the operator.

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A COMPARATIVE HISTOLOGIC STUDY OF HUMAN PERIODONTAL STRUCTURES UTILIZING FLUORESCENCE AND VISIBLE LIGHT MICROSCOPY

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INTRODUCTION

THE technique of detection and localization of certain substances in tissues and cells by virtue of their fluorescent properties when subjected to ultraviolet irradiation is finding increasing application. Some materials have sufficient intrinsic or autofluorescence for direct visual examination; others can be made to fluoresce by impregnating them with fluorescent chemicals called fluorochromes or fluors. For the most part, the use of fluors is limited to purely morphological studies, without regard to their chemical nature. Certain cellular elements exhibit selective absorption of the fluorochromes, depending in part on tissue preparation and pH of stain.

In many cases, fluorescence microscopy has proved itself capable of yielding more clearly differentiated histologic pictures than can be achieved by ordinary methods. It is a specialized method designed to supplement, not supersede, other histologic methods.

The primary purpose of this investigation was to determine the usefulness of fluorescence microscopy in histopathologic studies of the supporting structures of the teeth. Critical comparisons were made with the visible light microscope using conventional staining techniques. Collateral studies were made of the maturation of oral epithelium in benign dyskeratosis and frank malignancy, fluorescent dye penetration of human gingiva, and exfoliative cytology of oral epithelium, and will be reported in later publications. Numerous fluorochrome dyes were evaluated at various concentrations and pH values to determine their usefulness when applied to various types of biologic material of interest to the dental researcher.

MATERIAL AND METHODS

A. *The Apparatus.*—The essentials required for satisfactory fluorescence microscopy, utilizing long wave length ultraviolet radiation, are an intense

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source of radiation in the near ultraviolet, a system of filters to selectively pass the desired wave length of radiation, and a conventional monocular laboratory microscope. The silver substage mirror should be replaced with one of aluminized front surface construction.

The source of radiation used was an AH-4 General Electric high pressure mercury vapor lamp of 100 watt capillary type, operating at 130 volts and 0.86 ampere. This bulb has a total output of 3,500 lumens, with strong radiation in the violet and ultraviolet regions, particularly at 3,650 angstrom units. Starting the lamp requires a special reactive ballast transformer* to permit A. C. operation. The lamp was set in a housing chamber† and centered in relation to the reflecting mirror behind it. The distance from the housing lens to the lamp is adjustable and the lamp should be placed at the most efficient point by trial observations. Focusing can be critical and the use of a crayon line or bright spot of fluor may be helpful.[9] After lamp adjustments were made, the lamp housing and microscope were placed on a board mount and secured in position to permit rapid use and maintenance of constant working distances.

The lamp housing described carries an adjustable holder for filters. Two filters were placed at the lamp housing. The first‡ was designed to remove visible radiation, whereas the second§ removed infrared and red radiation. A third filter‖ was inserted into the microscope ocular to remove stray ultraviolet radiation. A semidarkened room provides ideal working conditions.

There are no filters available at present which transmit untraviolet radiation and completely absorb the visible light.[8] However, there are varieties of filters which transmit the ultraviolet, violet, and red more or less freely and which absorb strongly in the blue, green, and yellow regions of the visible spectrum. These filters, like all others, differ greatly among themselves both in characteristic absorption and in thickness. Considerable technical information is available on filters used in this type of research.[5, 10]

Photomicrographs were made on Daylight Kodachrome and Panatomic-X black and white film. A 35-millimeter single lens reflex type camera was attached to the microscope with a Novoflex bellows and microscope adapter ring. A highly sensitive photovoltmeter¶ was used to calibrate the amount of light energy being emitted at the microscope exit. Records were made of many light readings at the time of film exposure. Trial black and white strips were made to determine a uniform pattern of exposures at various microscopic magnifications.

Because of the low intensity of the fluorescent image, exposures are much longer than for normal photography. Exposures varied from 5 to 15 minutes

*American Optical, Model 395T.
†American Optical, Model 390.
‡No. 5840, 2-inch red-ultra, Corning Glass Co.
§A 2-inch tissue culture flask, ⅛ inch thick, containing an 8 per cent aqueous solution of copper sulfate and 3 drops of concentrated sulfuric acid.
‖Wratten 2-B, Series 4, Eastman Kodak Co.
¶Model 514 M, Photovolt Corp.

with Panatomic-X. Kodachrome exposures were usually 15 to 20 minutes. Some high powers were extended to 45 minutes with good success. Radiation measurements with the voltmeter were not found useful in determining photographic exposures.

At high magnification it was often necessary to reflect visible light through the microscope in order to obtain critical focusing of the specimen on the ground glass viewer of the camera.

B. *Preparation of Material.*—Upper anterior teeth and associated periodontal structures were obtained from patients who were undergoing full mouth extractions and ridge preparation for dentures. Extreme care was taken not to disturb the exact relation of the tooth and wedge of supporting tissue being removed. The specimen was fixed immediately in 10 per cent neutral formalin, as this solution does not affect secondary fluorochroming procedures. Previous workers have reported that fixatives containing salts of heavy metals fluoresce when subjected to ultraviolet radiation.[2, 11]

Decalcification was carried out with 5 per cent nitric acid. The sections were prepared for paraffin embedding according to usual technics. Two hundred sections from five specimens were prepared and attached to regular glass slides, using a starch solution rather than albumen as an adhesive, as the latter is known to exhibit fluorescent properties.[12]

Prior to staining, the paraffin sections were placed in xylol for 20 minutes. They were then passed through absolute alcohol, 95 per cent alcohol, and 70 per cent alcohol for one minute each. After 10 minutes in distilled water, fluorochromation was performed.

C. *Staining.*—A whole series of anodic, cathodic, and electroneutral fluorochromes were tested at various pH values ranging from 1.4 to 10.0. The fluors used in this study were as follows:

Acridine orange	Acid fuchsin
Acridine yellow	Acriflavine
Berberine sulfate	Thioflavine
Fluorescein	Congo red
Rhodamine B	Neutral red

For complete technical data on the color index, type, colors, uses, and source of supply, the reader is referred to an appropriate text.[14] The dyes were used in concentration from 1:1,000 to 1:10,000 in distilled water or buffer solutions at the respective pH desired. Table I provides technical data concerning the preparation of the buffers. During the early part of the investigation, several buffers in the low pH range caused precipitates with the fluor. This was overcome by changing the method of preparation in every instance. Staining time varied from ½ to 20 minutes. The duration of staining did not seem to have as much effect as the strength of the stain. The 1:5,000 concentration proved effective. Fresh solutions were prepared from a supply bottle of concentrate every few days.

If the section was to be stained in combination with a buffer solution, it was immersed in the proper buffer for 2 minutes, so the tissues might become

TABLE I
BUFFER SOLUTIONS AND SYSTEM USED IN PREPARATION
(CLARKE & LOOBES STANDARD BUFFERS)

pH	SYSTEM USED
1.4	0.2N HCl and 0.2N KCl
2.2	0.2N HCl and 0.2N KCl
3.6	M/5 acetic acid and M/5 sodium acetate
4.4	M/5 acetic acid and M/5 sodium acetate
5.0	M/15 monopotassium and disodium phosphates
6.0	NaOH and KH2PO4
7.0	NaOH and KH2PO4
8.0	NaOH and H3BO3
10.0	NaOH and H3BO3

the same value as the buffer. It was removed and stained with a dye solution of similar pH. Usually a concentration of 1:5,000 was employed for 2 to three minutes. With some fluorochromes, a longer staining time was employed.

Excess stain was washed from the specimen with buffer solution or distilled water. A few drops of buffer or distilled water was applied. The cover slip was pressed firmly to place with blotter paper. The edges were then sealed with hot paraffin wax.

Best results were obtained by using fresh wet sections, and examining them the same day of preparation. Sections were stored for weeks in Coplin jars with distilled water. It was noted, however, that many of these specimens lost some of their original fluorescence. This was apparently due to alterations in the dye concentration.

Various types of permanent mounts have been described.[3, 13] Isobutyl methacrylate was prepared for this purpose. However, the need to dry the tissues prior to application of such a liquid plastic material revealed gross artefacts, when compared with fresh specimens. The refractive index of methacrylate is preferable to water for high powers, but the degree of morphological distortion rendered it impractical. Some of these permanent mounts were examined after 6 months. The dye color appeared to remain intact over this period, but when ultraviolet irradiation was applied, a rapid fluorescence fatigue was observed. This fatigue was not as marked on fresh or stored wet specimens. Long photographic exposures were mandatory. Fluorescence fatigue thereby becomes a serious problem in registration of color.

Oil and glycerin were tried as mounting media. Color values were excellent with oil, yet the cells appeared shrunken and distorted in many instances. Glycerin exhibited marked fluorescence and was discarded.

Comparative studies were made using staining methods routinely used in visible light microscopy, namely, hematoxylin and eosin and Mallory's triple connective tissue stain.

THE EXPERIMENTAL RESULTS

The periodontal structures were observed in their natural state (primary fluorescence) before addition of a fluorochrome. Differentiation of tissue or cell

types was not satisfactory prior to staining and fluorescence colors appeared weak. Table II gives a general summary of the primary fluorescence colors noted for the various tissue elements.

TABLE II
PRIMARY FLUORESCENCE COLORS OF THE PERIODONTAL STRUCTURES
(DECALCIFIED PARAFFIN SECTIONS, MOUNTED IN DISTILLED WATER)

Gingival Epithelium	cytoplasm—gray-blue nuclei—dark purple-blue keratin—light blue
Connective Tissue Fibers	fibers—purple nuclei—light blue
Free Blood Cells and Macrophages	light blue
Vascular Walls and Contents	walls—same as connective tissue fibers contents—light blue
Cementum	robin's-egg blue
Secondary or Dental Cuticle	light blue
Alveolar Bone	dark gray-blue
Epithelial Rests	not distinguishable
Calculus	brownish-blue
Other	enamel matrix—gray-blue dentin—dark-blue

The entire series of fluorochromes was applied in varying pH solutions to determine the fluor of choice for the tissue elements comprising the periodontium. As noted by other workers, the basic fluor, acridine orange, was very useful for selective differentiation of tissue elements.[1, 7] Very subtle color differentiation was noted with this dye stuff at certain pH values, which was apparently related to the isoelectric points of these tissues. From pH 1.4 to 3.6, a weak greenish-blue color was noted. This is similar to the primary fluorescence of the tissue. At pH 4.4, a bright orange fluorescence was noted on structures that show heavier absorption (e.g., blood cells, cuticular structures, nuclei, dentin-cementum junction). At pH 5.0 and 6.0, the selectivity was about the same except for increased intensity of color. Table III presents the secondary fluorescence colors noted at pH 5.0. This table is a representative cross section of observations from many slides. From pH 6.0 to pH 10.0, all structures absorbed heavily, giving a brilliant fluorescence with less differentiation of elements. This evidently corresponds to the concentration effect of the dye when in molecular solution rather than dissociated into ions.[15]

Berberine sulfate exhibited similar staining properties to those shown by acridine orange, the fluorescence image being an intense yellow. However, differential selectivity at varying pH values was not as definite.

An interesting combination of fluors was discovered after many trials. Congo red at pH 6.0 was applied for 2 minutes. This was rinsed in distilled water, after which berberine sulfate was applied for 2 minutes at the same

TABLE III
SECONDARY FLUORESCENCE COLORS OF THE PERIODONTAL STRUCTURES
(DECALCIFIED PARAFFIN SECTIONS, MOUNTED IN pH 5.0 BUFFER SOLUTION AFTER STAINING WITH ACRIDINE ORANGE 1:5,000, pH 5.0)

Gingival Epithelium	cytoplasm—strong, yellow-orange nuclei—medium, greenish-yellow keratin—strong, clear yellow
Connective Tissue Fibers	medium purplish-brown fibers; faint orange nuclei in some areas
Free Blood Cells and Macrophages	strong whitish-orange
Vascular Walls and Contents	endothelial nuclei—strong yellow
Cementum	medium orange-green; precementum—green; cementodentin junction—bright, clear orange
Secondary Cuticle	strong, clear orange
Alveolar Bone	medium green with medium orange striations denoting lamellar apposition; osteoid—strong orange
Epithelial Rests	strong, yellow-orange
Calculus	strong, brownish-orange
Other	enamel matrix—yellow-white; dentin—medium greenish-orange; predentin—clear green; odontoblasts—light tan

pH. This combination stained the connective tissue fibers a deep blue-red, making an excellent contrast to the bright yellow epithelial and cuticular structures. The blood cells fluoresced a bright yellow-white. Lamellar patterns of cortical bone were readily noticeable. The marrow elements fluoresced bright yellow to blue-gray. Cementum appeared similar to bone except for a more speckled appearance.

The remaining fluors did not exhibit suitable differentiation; several exhibited a brilliant over-all fluorescence (fluorescein, acriflavine, rhodamine B).

The basis of fluorescence microscopy is the selectivity of tissues to the fluorochrome utilized and its subsequent color image in the microscope. Color plates, therefore, would be very helpful in describing the phenomenon. However, certain features can be distinguished, as will be noted in the following black and white photomicrographs from both the fluorescence microscope and visible light microscope.

Fig. 1 is a fluorescence photomicrograph of tissue from the gingival sulcus, stained with acridine orange at pH 5.0, registered on black and white film. Epithelial and associated cuticular structures are easily differentiated from the underlying mesenchymal elements. The color and intensity of this image is described in Table III. Fig. 2 illustrates at higher magnification the cuticular portion of the epithelial attachment in relation to the cementum. The cuticle fluoresces a strong clear orange.

Another specimen of attached gingiva, sulcus, and tooth is illustrated in Fig. 3. Here the cuticular attachment to the cementoenamel junction may be

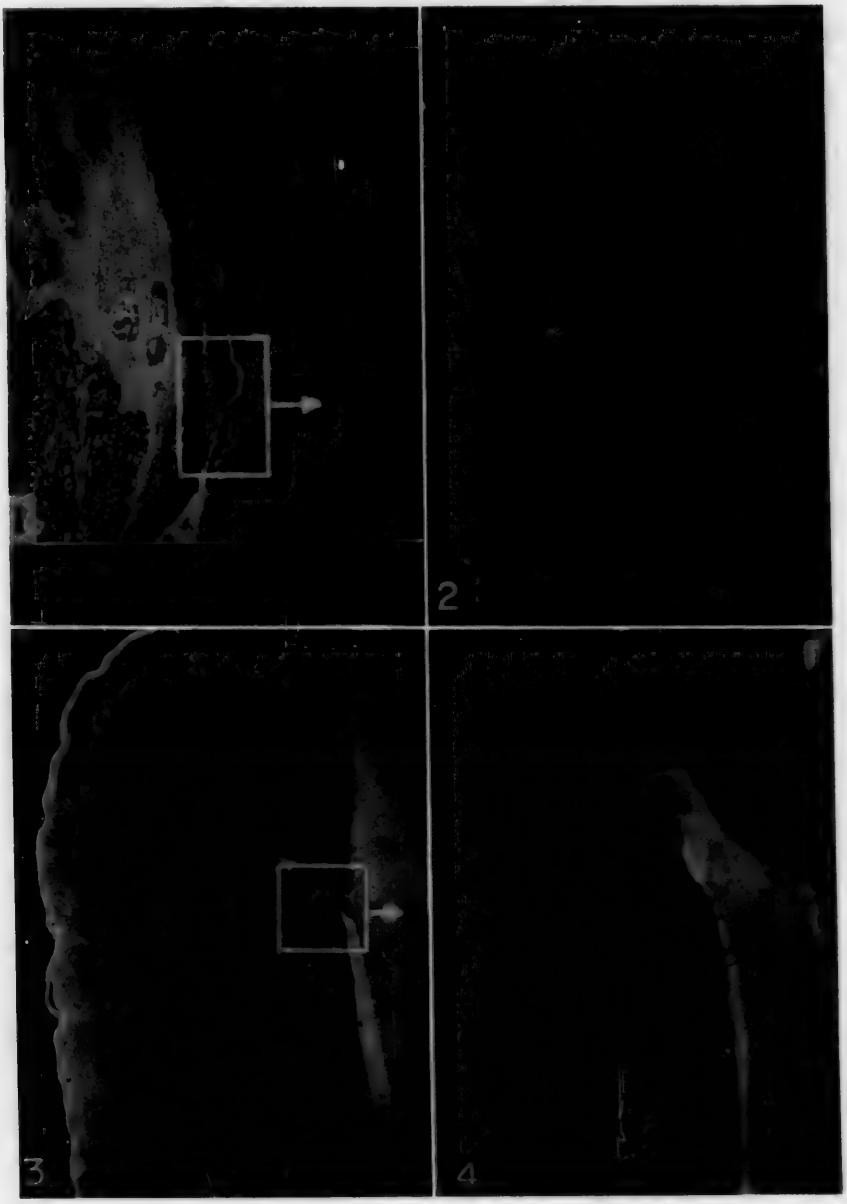

Fig. 1.—Gingival sulcus. Note torn epithelial attachment cells with cuticle adhering to exposed portion of dentin between enamel space and cementum. (Acridine orange, pH 5.0; orig. mag. ×100; fluorescence microscope.)

Fig. 2.—High power of enclosed area from Fig. 1. Note cuticular material adhering to tooth. (Orig. mag. ×440.)

Fig. 3.—Gingival sulcus. Note cementoenamel junction, cuticle extending upward denoting outline of enamel prior to decalcification, torn epithelial cells: (Acridine orange, pH 5.0; orig. mag. ×40; fluorescence microscope.)

Fig. 4.—High power of enclosed area from Fig. 3. Note selectivity of remnants of enamel matrix for the fluorochrome. (Orig. mag. ×440.)

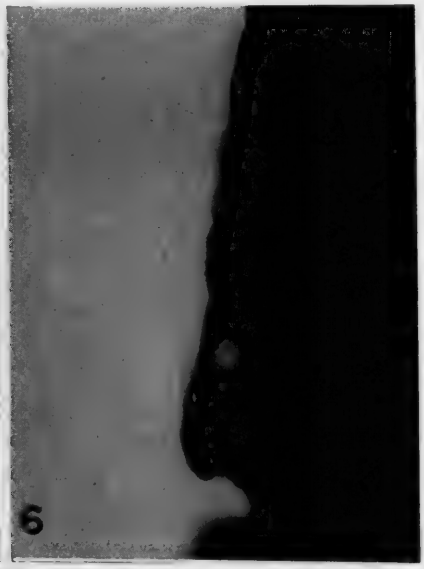

Fig. 5.—Area apical to bottom of gingival sulcus. Epithelial attachment torn in processing. Note cementoenamel junction with epithelial cells outlining former enamel surface. (Hematoxylin and eosin; orig. mag. ×100; visible light microscope.)

Fig. 6.—High power of enclosed area from Fig. 5. The most apical extent of the epithelial attachment cells. Note presence of flattened nuclei against the cementum. (Orig. mag. ×440.)

Fig. 7.—Gingival sulcus. Epithelial attachment located on cementum. Note ulceration of pocket epithelium and inflammatory infiltration of corium. (Hematoxylin and eosin; orig. mag. ×100; visible light microscope.)

Fig. 8.—High power of another portion of section used in Fig. 7. Note flattened nuclei adjacent to cementum and projections of cuticular material extending into the irregularities of the cementum. (Orig. mag. ×440.)

seen very well. The epithelium of the sulcus is ulcerated and disorganized. A chronic inflammatory reaction is present in the corium. Fig. 4 shows a higher power of the cementoenamel junction, the secondary cuticle, and remnants of the enamel matrix.

Fig. 5 illustrates another periodontal section stained with hematoxylin and eosin and photographed in visible light. The epithelial attachment has been torn from the cementum in processing. Fig. 6 is a high power of the epithelial attachment cells nearest to the apex noted in Fig. 5. Figs. 7 and 8 illustrate a similar specimen prepared for the visible light microscope with hematoxylin and eosin staining. The identification of both cytoplasmic and nuclear elements is more specific. The increased radiation, that is available with visible light, permits greater resolution of fine cellular details than was realized with fluorescence microscopy of similar sections.

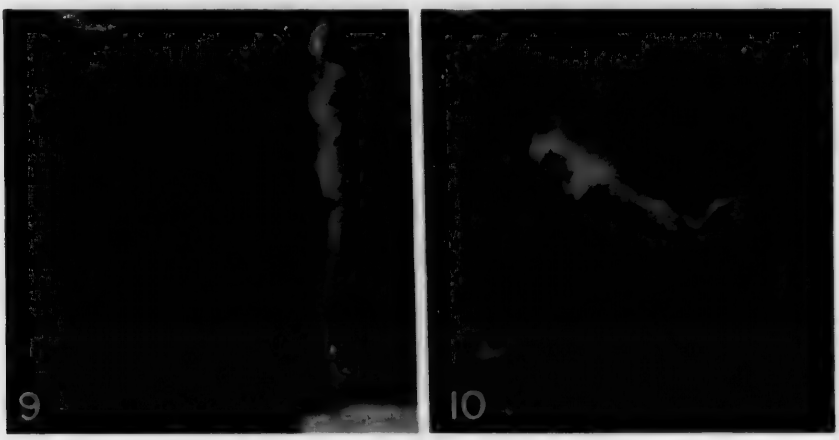

Fig. 9.—Nest of epithelial cells in the periodontal membrane. Dark nuclear halo surrounded by moderately bright yellow fluorescence of cytoplasm. (Acridine orange, pH 5.0; orig. mag. ×440; fluorescence microscope.)

Fig. 10.—Nest of epithelial cells in the periodontal membrane. Cementum surface on the right. (Hematoxylin and eosin; orig. mag. ×440; visible light microscope.)

A nest of epithelial cells in the periodontal membrane is shown in Fig. 9. These stand out rather brightly in most instances, especially when tending to be more keratinized or calcified. Fig. 10 illustrates a similar epithelial rest prepared for visible light observation. Here again, nuclear and cytoplasmic detail is more clearly differentiated. However, the selectivity of these epithelial cells to fluorochromation renders them readily noticeable when viewed against the darker background of the connective tissue structures comprising the periodontal membrane.

A calcified pulp stone and vascular channels of the pulp are noted in Fig. 11. The stone fluoresced a brilliant orange. The vascular contents exhibited a bright blue-white color. This selective absorption of the fluorochrome by the blood cells gave the impression that pulp circulatory studies could be efficiently performed with this technic.

DISCUSSION

An attempt was made to correlate morphology and fluorescence staining colors with the clinical diagnosis made prior to the surgical removal of the tooth and attached periodontal structures. This correlation was found to be impossible, as interpretation of minute cellular elements, especially of the soft connective tissues, was found to be too speculative. A critical evaluation of stains and staining technics necessary for improved secondary fluorescence images was carried out to provide useful information for studies of the several tissue types of the tooth and attached periodontium.

Fig. 11.—Pulp stone and vascular channels of adult pulp. Calcified stone fluoresces brilliant orange; the blood cells blue-white. (Acridine orange, pH 5.0; orig. mag. ×440; fluorescence microscope.)

There are certain advantages to the fluorescence technic. The staining procedure can be quickly performed. Stain absorption is easily regulated by adjusting the pH of the staining soluation. The resulting colors also depend upon dye concentration. Usually it is only necessary to use a single fluorescent stain in order to produce a structural differentiation, which is invariably far superior to that which would have been obtained from using a single diachrome. In some instances, the differentiation obtained is superior to multiple diachromatic methods.

As noted earlier, primary fluorescence of unstained sections was not significant. However. this effect must always be taken into consideration, when evaluating secondary fluorescence colors. For studies involving penetration of fluorescent chemicals, intravital injections, or localization of pigments, a complete knowledge of the primary fluorescence of the tissues under observation is imperative.

It is generally believed that different kinds of tissues glow in different colors, depending upon their chemical structure.[6] Further, the color and intensity of fluorescence depends upon the relative basophilia and acidophilia of the individual cellular elements, and upon the nature of the fluorochrome. This, of course, explains the difference in absorption noted at various pH values. Information as to the chemical nature of the cellular elements under study remains speculative.

The problems of interpreting the resultant colors after fluorochromation are many. The following factors must be considered: the thickness of the preparation, the dyestuff, the concentration and pH of the dyestuff, the source of radiation, filters, and the color value interpretation of the individual observer.

Attemps have been made by some workers to register fluorescence colors. Hals developed an elaborate color plate for isoelectric point determination of the developing tooth germ.[7] His registrations were made photographically using the same microscope, the same film, and uniform processing procedures. Bennett developed a master color circle in which all colors, their intensity, and hue would supposedly fit.[1]

Spectroscopic analysis of fluorescent images is probably the most logical approach to the problem. Undoubtedly, as sources of radiation improve, such measurement of fluorescence will be more practical. Dickson, Forziati, and Schoonover offer encouraging developments along these lines.[4]

The most outstanding feature of this investigation was the marked clarity of the epithelial attachment cuticle. The brightness of the fluorescence of this structure, in contrast to that of the adjacent epithelium and cementum, was very noticeable. The use of increased intensity of radiation together with thinner sections and different mounting media would permit higher resolution of this system.

SUMMARY

Human periodontal structures were sectioned and stained for histologic observations with both fluorescence and visible light microscopic technics.

Primary fluorescence of unstained sections of periodontal structures is unsatisfactory for histologic study.

Separate and combined fluorochromes were utilized at varying pH values to determine the most efficient technics applicable to this type of research. Fluorchromation of histologic sections of the periodontium reveals interesting secondary fluorescence colors. These colors differ according to pH values of the stain.

The attachment of the secondary cuticle to the cementum surface is clearly revealed by the fluorescence microscope. Similar sections prepared with hematoxylin and eosin and Mallory's triple connective tissue stains and examined by visible light microscopy do not show differentiation of the cuticle-cemental interface as well.

Morphological details of the cellular elements of connective tissues were better differentiated with conventional staining methods utilized in visible light microscopy.

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THE EFFECTS OF BETA-AMINOPROPIONITRILE UPON THE MOLAR TEETH OF THE RAT

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THE experimental studies of Geiger, Steenbock, and Parsons[1] and Ponseti and Shepard[2] have shown that certain connective tissues, and in particular bone, cartilage, and aortic wall, are greatly affected by the seeds of *Lathyrus odoratus*. Subsequent investigations identified the toxic factor in these seeds to be beta-aminopropionitrile.[2, 3, 4]

With the exception of enamel, the tissues of the tooth are of mesodermal origin. Since beta-aminopropionitrile is known to exert profound effects upon connective tissues, knowledge of its effects, if any, upon these types of mesodermal tissue would be of interest. In this paper the effects of beta-aminopropionitrile upon the molar teeth of the weanling rat will be reported.

MATERIALS AND METHODS

The materials used in this study are the same as those used in a previous investigation[5] in which the effects of beta-aminopropionitrile on certain oral structures were studied. Briefly, 70 male and female weanling rats were divided into control and experimental groups. All animals were fed, ad libitum, a synthetic diet; 0.2 per cent beta-aminopropionitrile† was added to the diet of the experimental rats. Fresh batches of diet were prepared every 7 to 10 days, and each time freshly distilled beta-aminopropionitrile was used.

Control and experimental animals were killed after 3, 6, 9, 13, 17, 23, and 28 days on the diet. On each of these days, 5 control and 5 experimental animals were used. The oral cavity was examined grossly and then one half of the mandible was fixed in Bouin's fluid, decalcified in a mixture of equal amounts of 45 per cent formic acid and 20 per cent sodium citrate, embedded in paraffin, sectioned and stained with hematoxylin and eosin, with van Gieson's stain, and Foot's silver stain.

By the use of an ocular micrometer the following measurements were made for the purpose of quantitative comparisons between the two groups of animals: (a) the length of the distal root of the first molar from the cementoenamel

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junction to the apical foramen, (b) the thickness of the dentin and predentin at the level of the cementoenamel junction, and (c) the greatest thickness of the cellular cementum. Only when the microscopic section was through the pulp chamber, root canal, and apical foramen were measurements made. The distal root of the first molar was preferred because it satisfied the above requirements in most of the sections. Since enamel formation is not a continuous process, the cementoenamel junction can be considered as a fixed point and the measurements are, therefore, comparable.

Fig. 1.—Apical area of first molar root of a 34-day-old normal rat. (Hematoxylin and eosin.)

OBSERVATIONS

Gross examination did not reveal any abnormalities of the enamel of the experimental rats. At the beginning of the experiment the first two molars had already erupted. It was noted that the third molar erupted at the same time in experimental and control animals (approximately on the thirty-fifth day).

Microscopically, no abnormalities could be observed in the molar teeth of the rats killed after 3 and 6 days on the diet. However, in the experimental rats killed on the thirteenth experimental day, changes were evident in the apical region of the root of the first and second molars. In normal rats of this age, both dentin and cellular cementum end at the root apex in a point, which is almost at the same level, the cellular cementum covering only the external surface of the dentin. From the apical end of the junction, between dentin and cellular cementum, the two-layered epithelial diaphragm arises. It projects in a horizontal direction, thus narrowing the apical foramen (Fig. 1). In the apical

area of the molars of the experimental animals, the dentin which normally does not contain cells but only cytoplasmic processes of the odontoblasts, did contain cells, presumably odontoblasts, which were enclosed within lacunae of the dentin matrix. In this respect this dentin resembled bone and may, therefore, be called osteodentin. It contained few and irregularly arranged dentinal tubules and its fibrils as revealed by the silver stain were also haphazardly arranged. It was thicker than the normal dentin at the same location and its pulpal surface was very irregular and was lined by haphazardly arranged odontoblasts. Most of them retained their elongated form and appeared to be slightly increased in number and to have an increased cytoplasmic basophilia.

Fig. 2.—Apical area of a first molar root of a 34-day-old rat receiving beta-aminopropionitrile for 13 days. The dentin contains cells, the cellular cementum covers the apical surface of the dentin, and a recognizable epithelial diaphragm is absent. (Hematoxylin and eosin.)

A recognizable epithelial diaphragm was absent and the cellular cementum covered not only the periodontal but also the apical surface of the thickened dentin. Thus, the apex of the root was formed by cementum only (Fig. 2). These changes were accompanied by a possible decrease in root length and in the thickness of cellular cementum. For some reason, which we are unable to explain, the differences in root length and thickness of cellular cementum could not be seen in the rats killed after 23 days on the diet (Table I).

We have reported that in experimental lathyrism the fibers of the periodontal membrane exhibit a loss of their coarse fibrillar appearance and characteristic orientation. Instead they appear to consist of very fine fibrils, which

TABLE I

EFFECTS OF BETA-AMINOPROPIONITRILE ON ROOT LENGTH AND THICKNESS OF CEMENTUM, DENTIN, AND PREDENTIN

DAYS ON DIET EXPTL.	LENGTH OF DISTAL ROOT OF FIRST MOLAR IN MM.		THICKNESS OF CELLULAR CEMENTUM IN MICRONS*		THICKNESS OF DENTIN AT CEJ† IN MICRONS		THICKNESS OF PREDENTIN AT CEJ† IN MICRONS	
	CONTROL	EXPTL.	CONTROL	EXPTL.	CONTROL	EXPTL.	CONTROL	EXPTL.
3	1.3	1.3			136	136	8	6
	1.4	1.3			143	136	11	8
	1.4	1.3			150	150	11	8
		1.3				150		8
		1.4				170		8
6	1.4	1.2			170	163	6	6
	1.4	1.3			170	170	6	6
	1.5	1.3			170	170	8	6
13	1.6	1.3	79	47	218	204	5	5
	1.6	1.5	83	47	245	218	5	5
	1.7	1.5	86	55	245	218	8	6
		1.5		55		231		8
23	1.4	1.4	58	55	211	211	5	5
	1.5	1.6	60	82	272	238	6	5
	1.6	1.7	79	86	286	306	6	
28	1.9	1.3	77	50	224	218	3	3
	2.0	1.6	86	63	252	258	5	6
	2.0	1.7	93	63	265	272	6	6
	2.1	1.8	107	74	326	279	6	

*In rats sacrificed on the third and sixth experimental day, cellular cementum was either present in very small amounts or entirely absent.

†CEJ—Cementoenamel junction.

have no definite orientation and are embedded in increased amounts of an amorphous eosinophilic material surrounded by palisading fibroblasts[5] (see Fig. 6). With the silver and van Gieson stains, these areas gave reactions consistent with the presence of collagen fibers. We do not know whether this material is related to bone or cementum matrix.

In contrast to the above observations, the structure of the dentin in the coronal part of the roots and in the crown of the first and second molars of the experimental rats was similar to those of the controls (Fig. 3). The thickness of the dentin and predentin at the cementoenamel junction was the same in both groups of animals (Table I). Similarly, the hematoxylin and eosin, van Gieson, and silver stains revealed no abnormality in the dental pulp. The odontoblasts, pulpal fibroblasts, and the argyrophilic fibers were normal in numbers, appearance, and arrangement.

The changes seen in the apical region of the roots of the first and second molars of the experimental rats were absent in the third molars of all groups of these animals. The dentin, cementum, and epithelial diaphragm, and the odontoblasts, pulpal fibroblasts, and argyrophilic fibers of the pulp of these teeth did not present any abnormality (Fig. 4). The periodontal membrane of the third molar was also free of any change.

Since enamel formation of the molar teeth was completed before the beginning of the feeding, the effects, if any, of beta-aminopropionitrile on amelogenesis could not be studied.

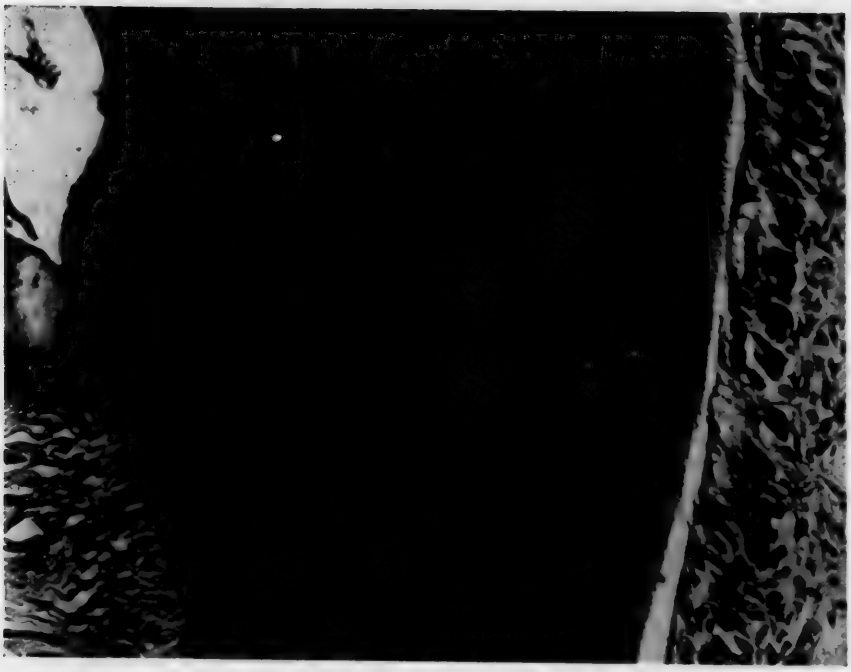

Fig. 3.—Dentin and odontoblasts of a first molar at the level of the cementoenamel junction of a 49-day-old rat receiving beta-aminopropionitrile for 28 days. The pulpal half of the dentin was probably formed during the experimental period. The dentin and the odontoblasts appear normal. (Hematoxylin and eosin.)

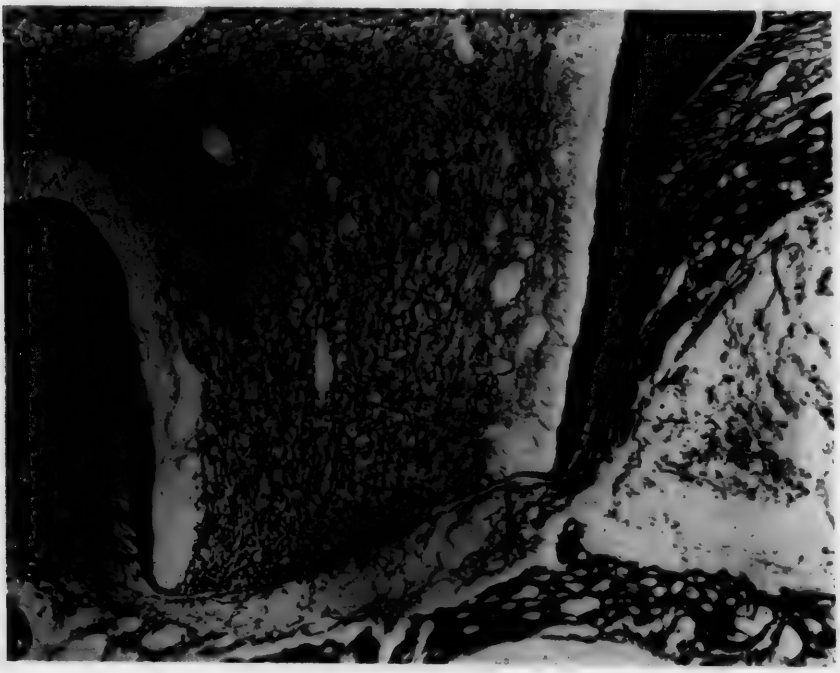

Fig. 4.—Third molar root of a 34-day-old rat receiving beta-aminopropionitrile for 13 days. The root was probably formed during the experimental period. The number and arrangement of the argyrophilic fibers of the pulp appear normal. (Silver stain.)

DISCUSSION

In contrast to bone, dentin and cementum, for some unknown reason, are normally spared from cellular resorption (with the exception of the shedding of the deciduous teeth) and are subject only to a continuous deposition, although its rate is highest in the young animal. On the submicroscopic level, however, radioisotope studies have offered evidence suggesting that an exchange of chemical substances does take place between the hard dental tissues and the adjacent fluid.[6, 7] This state of affairs permits accurate separation of pre-experimental from experimental tissue and, thus, allows better quantitative comparison of appositional growth and more accurate determination of the changes occurring in teeth than in any other organ of the body.

In this investigation, conditions permitted the study of the effect of beta-aminopropionitrile on connective tissue cells differentiated prior or during the experimental period, in the presence or absence of mechanical stress. They also permitted, as in the case of the root of the third molar, good separation between pre-experimental and experimental tissue.

In the apical region of the first and second molars the odontoblasts have most likely differentiated during the experimental period, and in the rats killed after 13 or more days on the diet have been under the influence of mechanical stress. It has been shown that the first and second molars of the rat are in functional occlusion on the twenty-fifth and twenty-eighth day, respectively.[8] It is interesting to note that changes were absent in experimental rats killed after periods of less than 13 days on the diet. This is at a time when the first and second molars were not supposed to be in functional occlusional. Since there is evidence indicating that mechanical stress plays an important role in the morphologic changes of lathyrism,[5, 9-11] it is possible that, in the presence of mechanical stress, beta-aminopropionitrile disturbs the normal interplay among epithelial proliferation, dentin, and cementum deposition. It is quite possible that a disturbance in the differentiation of the odontoblasts resulted in the production of abnormal dentin (osteodentin) (see Fig. 2).

The majority of odontoblasts present in the coronal parts of the roots and the pulp chamber of the first and second molars have differentiated before the initiation of the feeding and are protected by the calcified structures from any significant mechanical stress. Perusal of Table I suggests that in the last sacrificed group of experimental rats, approximately the pulpal half of the dentin at the level of the cementoenamel junction was formed during the experimental period. Our observations suggest that in the absence of mechanical stress, beta-aminopropionitrile does not appear to have any effect upon already differentiated odontoblasts and pulpal fibroblasts, or on the structure of the dentin produced by them in response to the usual "growth stimulus" (see Fig. 3). Also, under these conditions the rate of deposition of dentin does not appear to be affected (Table I).

The formation of the roots of the third molars had not started at the beginning of the experimental period. It may, therefore, be said that the dentin, cementum, pulp, and periodontal membrane of the third molar roots of the

rats were formed during the experimental period and are free of any pre-experimental tissue. It is quite possible that the third molars of all groups of experimental rats had never been in functional occlusion.[5] Our observations suggest that in the absence of mechanical stress, beta-aminopropionitrile has no effect upon the differentiation of odontoblasts and fibroblasts, and the production of argyrophilic and collagen fibers in the weanling rat in response to "growth stimulus" (Figs. 4, 5, 6). Although there is sufficient evidence suggesting that

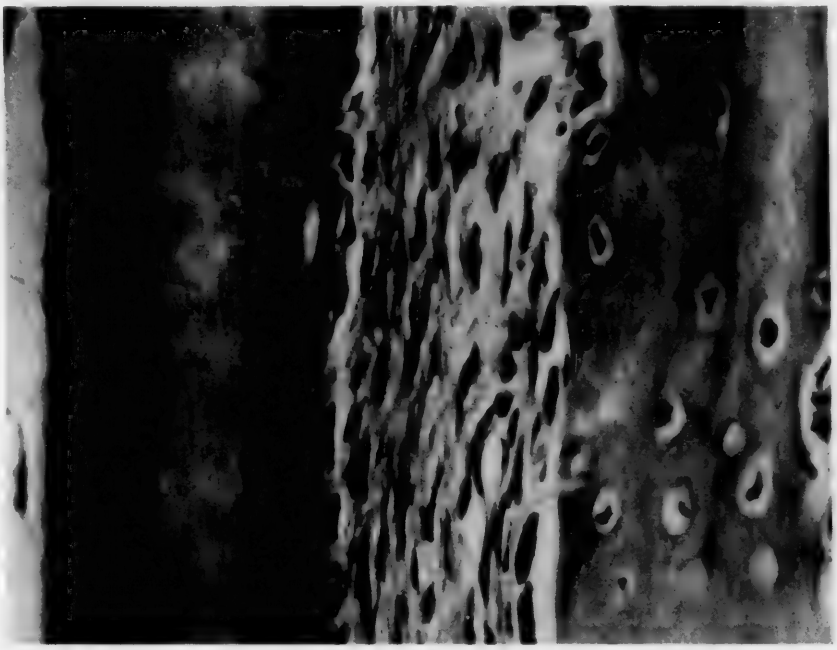

Fig. 5.—Normal appearing periodontal membrane of the third molar of a 49-day-old rat receiving beta-aminopropionitrile for 28 days. (Hematoxylin and eosin.)

mechanical stress is important in the alterations of lathyrism, when attempting to interpret the absence of changes in the periodontal membrane and pulp of the erupting third molars, it may be worth while to take into consideration the composition of the periodontal and pulpal connective tissue. In the control and experimental rats used in this experiment, the periodontal connective tissue of the third molars was cellular and contained only a few and thin collagen bundles. The fibers of the pulp were exclusively argyrophilic. It is possible that the composition of the periodontal and pulpal connective tissue of the third molars of these young rats may be related to the absence of changes in these tissues. If beta-aminopropionitrile affects the later stages of the formation of the intercellular substances of connective tissue, such an interpretation is a reasonable one.

Recent studies[11-15] suggest that in experimental lathyrism there is a disturbance in collagen synthesis and in the proliferation and differentiation of

fibroblasts. Whether this disturbance in collagen synthesis is a qualitative one or only a quantitative one remains to be seen. All these studies have in common the fact that collagen formation and fibroblastic proliferation and differentiation were studied in response to some sort of injury, such as wounding or injection of croton oil. Our observations suggest that in experimental lathyrism and in response to "growth stimulus" only, no changes can be seen (at least by the methods we employed) on the differentiation of odontoblasts and fibroblasts, and on the production of argyrophilic fibers, collagen fibers, dentin, and cementum. However, a change, as yet of unknown nature, must be present, because once an injury is produced the response is most abnormal.

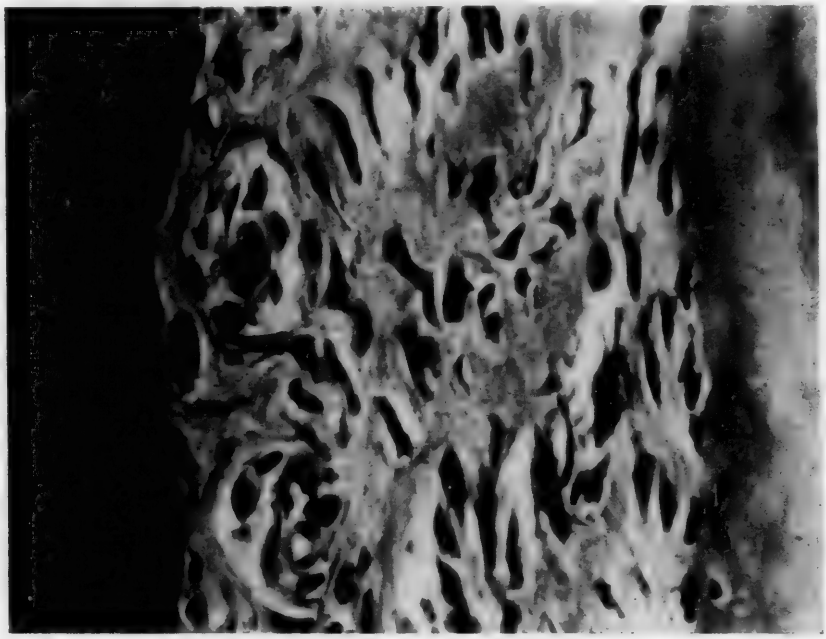

Fig. 6.—Periodontal membrane of second molar. Great abnormalities are present. Photomicrograph taken from the same microscopic section as that of Fig. 5. (Hematoxylin and eosin.)

SUMMARY

1. The effects of a diet containing 0.2 per cent beta-aminopropionitrile on the molar teeth of weanling male and female rats were studied. The following observations were made:

a. In the apical regions of the first and second molars of the experimental rats killed after the thirteenth day on the diet, an abnormal type of dentin (osteodentin) was deposited, a recognizable epithelial diaphragm was absent, and the apex of the root was formed by the cementum only. The rate of root elongation and the thickness of cementum appeared to be decreased.

b. In the coronal parts of the roots and in the crown of the first and second molars of the experimental rats, the structure of the dentin, and the number,

appearance, and arrangement of the odontoblasts, pulpal fibroblasts, and argyrophilic fibers of the pulp were found to be normal. At the level of the cementoenamel junction, the thickness of the dentin and predentin was the same in both groups of animals.

c. The dentin, cementum and epithelial diaphragm, the odontoblasts, and argyrophilic fibers of the pulp, and the collagen fibers and fibroblasts of the periodontal membrane of the third molars of the experimental rats did not present any abnormality.

2. The significance of these observations in relationship to the effects of beta-aminopropionitrile on cell differentiation and fiber formation in the connective tissues is discussed.

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RADIATION OUTPUT OF DENTAL X-RAY UNITS

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DENTAL radiographic technics are dependent upon the radiation generating capabilities of dental x-ray equipment. The inconsistency of the success of good radiographic technics in different dental offices has prompted the investigation of the radiation output of dental x-ray units.

PROCEDURE

Equipment.—The x-ray units used in this study were in the offices of practicing dentists. They ranged in age from one week to approximately 20 years, with the average age of 7 years. Each unit was considered to be in good working order by the dentist. They were the products of four different manufacturers which included the most universally used types of units. The testing equipment consisted of a roentgen meter* with two ionization chambers, one of 25 r capacity and one of 100 r capacity. The unit was calibrated approximately 10 days prior to its use in this study. The additional equipment consisted of a lead shield 10 inches square and ¼ inch in thickness, a steel tape measure, a stop watch, and tabulation forms.

Details of Procedure.—The procedures used in performing the tests were carried out in eleven (11) steps.

1. The line voltage reading was checked and the transformer control adjusted to a rating of 110 volts during unit operation.

2. The milliamperage reading was checked and set at 10 Ma. during unit operation. Those units not having a numerical calibration on the milliammeter were set to operate within the recommended range.

3. The radiation aperture at the tube head for auxiliary filtration of primary radiation was checked.

4. The kilovoltage of those units equipped with a KV control was checked. KV control was set at 65 kv. during unit operation.

5. The accuracy of the timer was checked with a stop watch.

6. The ionization chamber was charged to zero setting and positioned on the face of the lead shield supported below the tube head.

7. The short cone tip-meter distance was adjusted. The points of measurement of this distance setting was the tip of the short cone and the top of the chamber surface, with the chamber lying horizontal on the lead shield.

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*Victoreen, Model 70.

Four distance settings were made and four exposures made: No. 1 setting was 1 inch; No. 2 setting, 4 inches; No. 3 setting, 10 inches; and No. 4 setting, 17 inches.

The approximate tube-target meter distance for setting: No. 1 was 8 inches; No. 2, 11 inches; No. 3, 17 inches; and No. 4, 24 inches.

8. Each exposure was 10 seconds with 10 Ma. reading and 65 kv. reading on units having a KV recording. Two separate exposures of 10 seconds each were made at each distance setting to recheck for error.

9. The charge of the ionization chamber was read and results recorded.

10. The ionization chamber was recharged and subsequent exposures and recordings were made.

11. Pertinent data of x-ray units such as make, model, serial number, age of unit, and frequency of use were recorded.

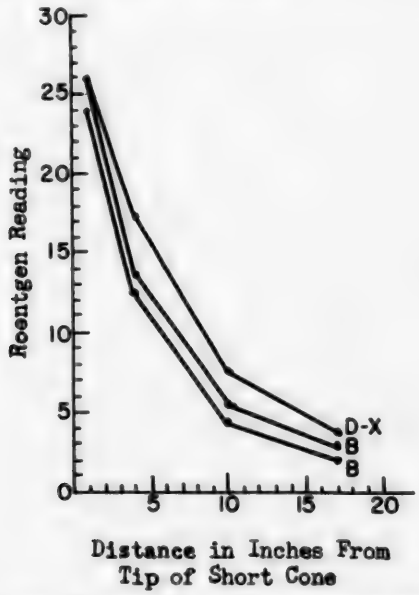

Fig. 1.—X-radiation output of Group A, dental x-ray units, operating at 65 kv., 10 Ma., for 10 seconds.

RESULTS AND CONCLUSIONS

A total of 14 dental x-ray units were used in the study. For diagrammatic purposes, the units were grouped according to manufacturer with Group A composed of three units, Group B three units, Group C seven units, and Group D one unit.

The tabulations of Group A (Fig. 1) are plotted graphically with the vertical column designating the roentgen readings and the horizontal column designating the distance in inches from the tip of the short cone to the roentgen meter. The roentgen output of two of the units at the one-inch distance was two of 26 r and one 24 r for a 10-second exposure at 10 Ma. The second

reading at the distance of 4 inches was 17.25 r, 13.5 r and 12.25 r, respectively. The third reading at a distance of 10 inches was 7.5 r, 5.5 r, and 4.5 r. The final reading at a distance of 17 inches was 3.75 r, 3 r, and 2 r. To evaluate these readings it must be emphasized that each exposure was made for 10 seconds at 10 Ma. at the same kilovoltage. The rapid fall of radiation detected in the first 4 inches of distance from the tip of the cone would indicate that much soft radiation was present in the primary beam and was dissipated in air. The use of these x-ray units with a short target-film distance technic would make the exposure time quite critical in regard to distances within the 3-inch range as well as expose the patient to much soft radiation. The output variance of these units throughout the scope of the graph would be sufficient to require different exposure times of each unit to produce the same radiographic results with all other factors being equal.

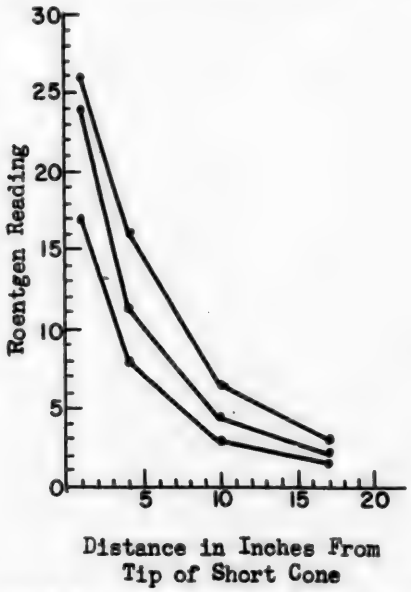

Fig. 2.—X-radiation output of Group B, dental x-ray units, operating at 65 kv., 10 Ma., for 10 seconds.

The tabulation of Group B is shown in Fig. 2. This group shows a much wider variance of radiation measurement at the one-inch and 4-inch distances, but tends to approximate the same findings as in Group A at the 10- and 17-inch distances. In using a short target-film distance technic with this group of x-ray units, a decidedly different exposure time would be required with each unit to produce the same radiographic results, all other factors being equal.

The tabulation of Group C (Figs. 3 and 4) show a considerable variance in radiation measurement and present a somewhat different pattern in that the measurement at the one-inch distance is low in comparison with Group

A and B, but falls in a comparable range in the 10- and 17-inch distance. This would indicate that the primary radiation beam is of a more uniform strength or wave length than the previous two groups, yet the variances in the individual unit output is such that each would produce a different radiographic result when all other conditions were equal.

The tabulation of Group D (Fig. 5), which involves only one unit, gave a pattern very similar to Group C.

To aid in the comparative evaluations of the x-ray units of the various manufacturers, each group was averaged and graphically plotted as shown in Fig. 6. The measurement of radiation of the four groups at the one-inch distance shows a wide range of radiation activity. The percentage variance of radiation measurement between the groups becomes less as the distance is increased. Using roentgen output as a basis for determining radiographic

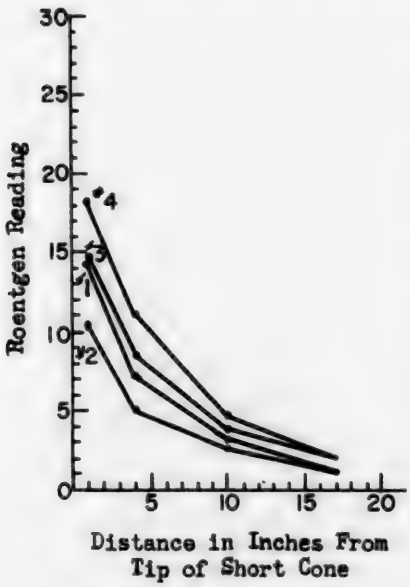

Fig. 3.

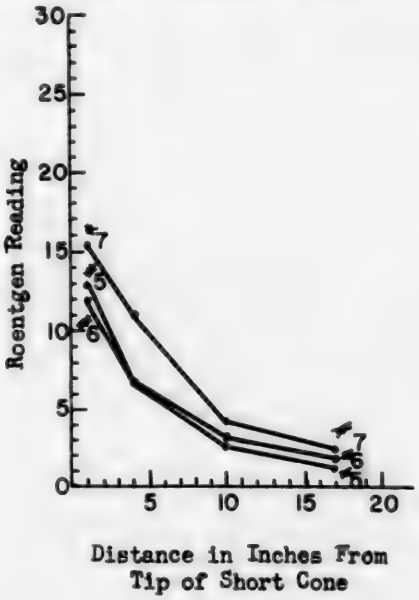

Fig. 4.

Fig. 3.—X-radiation output of Group C, dental x-ray units, operating at 65 kv., 10 Ma., for 10 seconds

Fig. 4.—Additional Group C dental units operating at 65 kv., 10 Ma., for 10 seconds.

exposure times, the operator using x-ray units of Group D would have to use a much greater exposure time than the operator using units of Group A, when using a short target-film distance to produce the same radiographic results. This would be true providing the strength of the primary radiation was approximately the same with all units.

No effort was made in this study to determine the quality of radiation in the primary beam. The same comparative ratio of radiation measurement between Group A and D is evident at the 10-inch distance. At the 17-inch

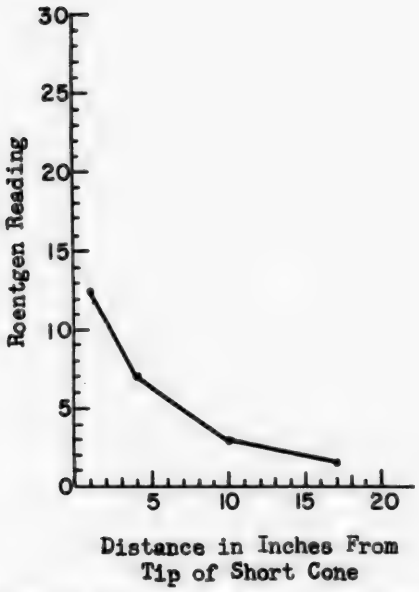

Fig. 5.—X-radiation output of Group D, dental x-ray units, operating at 65 kv., 10 Ma., for 10 seconds.

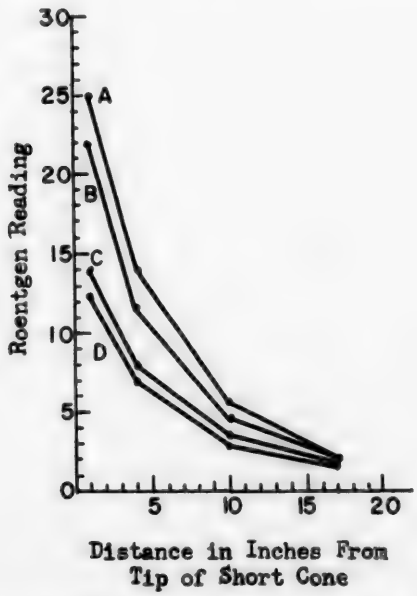

Fig. 6.—The average x-radiation output of each of the four groups of dental x-rays tested are designated by *A*, *B*, *C*, and *D*.

distance, the comparative ratio between the groups becomes smaller. This would indicate that the useful radiation of the primary beam is more nearly uniform at the 17-inch distance than at any of the closer distances for all of the x-ray units used in this study.

Many conclusions regarding technics and target-film distances can be assumed from these findings, but the capabilities of the dental x-ray units in producing sufficient radiation to produce good radiographic results at the greater distances must be considered.

Many inconsistencies of the dental x-ray units were encountered during this study. Most of the units required that the line voltage meter reading be set from 115 volts to 124 volts to produce a delivery voltage of 110 during unit operation. This was not found to be peculiar to any make of unit. The x-ray timers varied from 13 seconds to 6½ seconds for a 10-second setting. This great variance in timer operation required that a stop watch be used for making the exposures for this study. Most of the dentists did not know that their timers were incorrect. Some of the milliamperage meters on the units were not calibrated in numerical indications but provided only a range of milliamperage operation. Thus, the operator did not know the exact milliamperage being delivered. Only a few units provided a kilovoltage indicator and control. The dentists using the x-ray units without this indicator did not know the kilovoltage output of the unit.

SUMMARY

The findings of this study demonstrate that the dental x-ray units vary greatly in the roentgen output both in comparison of units of different manufacturers and somewhat less greatly in units of the same manufacturer. This variability is sufficient to defeat the efforts to standardize dental radiographic technics to produce uniform results.

THE EFFECT OF pH ON THE ANTIMICROBIAL ACTIVITY OF PARA-AMINOTOLUENE SULFONAMIDE

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FOR the past several years, we have employed para-aminotoluene sulfonamide as an endodontal antiseptic.[1] In our hands it has performed satisfactorily; however, there are occasions when it has failed, clinically, even in instances where laboratory bacteriologic tests indicated success should have been achieved. It is clear that some factor, or factors, is, in some fashion, suppressing the therapeutic activity of para-aminotoluene sulfonamide in certain cases of clinical use.

It is well known that certain common sulfonamides, such as sulfanilamide or sulfathiazole, exhibit a marked improvement in therapeutic activity when environmental pH is in proximity to 7.[2] Since the homosulfonamide para-aminotoluene sulfonamide (PATS) contains the characteristic $-SO_2NH_2$, it seems reasonable to assume that environmental pH may also affect the therapeutic behavior of this drug. If this were so, such a condition might explain in part some of the clinical failure experienced and, at the same time, suggest a suitable means for corrective action.

It is the purpose of this investigation to determine the effect of environmental pH on the antimicrobial activity of para-aminotoluene sulfonamide under laboratory conditions.

EXPERIMENTAL PROCEDURE

The general design of the project was to produce culture broth solutions containing PATS at concentrations varying between 5 per cent and 0 per cent, all at a given pH value. Such broth solutions were then inoculated with bacteria obtained from the Department of Endodontia. After 72 hours' incubation at 38° C., record was made of those solutions showing bacterial growth. Subcultures were made from all solutions into plain brain heart infusion broth, such subcultures were incubated 3 days at 38° C., and record made of those solutions showing growth or no growth. From these data, the bacteriostatic and bactericidal activity of the parent solutions could be determined.

The whole general experiment was then repeated for succeeding series of broth solutions at other pH values, the range investigated being from pH 3 to, and including, pH 9.

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Prior experience at this activity,[1] and the experience of others,[3] has shown that PATS is stable and soluble over a wide range of pH values. It was found, however, that broth solutions containing 1 per cent PATS were slightly unstable with regard to the dissolved protein of the broth. Such instability was increased slightly by increasing the PATS concentration, and by altering the pH in either direction from pH 7.2. For this reason, most of the solutions were prepared and used as soon as sterility checks were completed (3 days).

A typical series of broth solutions was prepared in the following manner. Ten grams of PATS were dissolved in 200 ml. of distilled water. This solution was then added to another containing 37.5 Gm. of dehydrated brain heart infusion powder (Difco) dissolved in 600 ml. of distilled water. The final volume was brought to 1,000 ml. with distilled water. This solution was then divided into eight equal portions. By means of small additions of concentrated phosphoric acid the pH of five such portions was adjusted, respectively, to values of 3.0, 4.0, 5.0, 6.0, and 7.0. The remaining portions were adjusted with small increments of concentrated sodium hydroxide to pH values of 7.4, 8.0, and 9.0.

A ninth portion of plain brain heart infusion broth was made and placed with the series to act as control.

All such broth solutions were immediately put through sterile Seitz filters, collected in sterile flasks, and aseptically transferred to sterile 7 ml. screw top culture tubes (5 ml. per tube). All solutions were then incubated at 38° C. for 3 days. If no change occurred in the solutions, it was assumed they were sterile. As will be seen later, this assumption is only partly valid in some of the instances.

The other series of experimental broth solutions were made up in a similar fashion but at PATS concentrations of 5, 4, 3, 2, 0.50, 0.10, 0.05, and 0.01 per cent.

Prior to inoculation with bacteria, sample vials from each PATS concentration and pH range were removed, tested for pH value, and discarded. This was necessary as occasionally a solution may have drifted from its expected pH value. Permissible variations in pH were limited to plus and minus 0.10 pH units.

From a number of bacterial cultures, obtained from the Department of Endodontia where they had been in incubation 72 hours, single 3 mm. loop transfers were made to fresh brain heart infusion broth and the latter incubated for 24 hours at 38° C. The gram-staining character and the general morphology of the bacterial inhabitants of each culture were determined on samples from these 24-hour growths. Such characteristics for the ten cultures used in this report are shown in Table I. Single 3 mm. loop transfers were then made in duplicate to the proper experimental culture solutions. All cultures included in this report, on continued transfers to plain brain heart infusion, exhibited no discernible change throughout the course of the project.

Observations were made after 24 and 72 hours' incubation at 38° C., although only the 72-hour observations will be reported here. Such observations consisted of noting the presence or absence of bacterial growth in the

experimental solutions and the control solutions. Absence of change in a solution was assumed to be indicative of no bacterial growth. Results typical of such observations are shown graphically in Fig. 1.

A single 3 mm. loop subculture was made aseptically from each vial to a properly identified vial of plain brain heart infusion broth, and the latter incubated in the fashion described for 3 days. If no change occurred in the subculture tubes associated with an experimental solution, but growth did occur in the subculture vials associated with the control tubes, then such

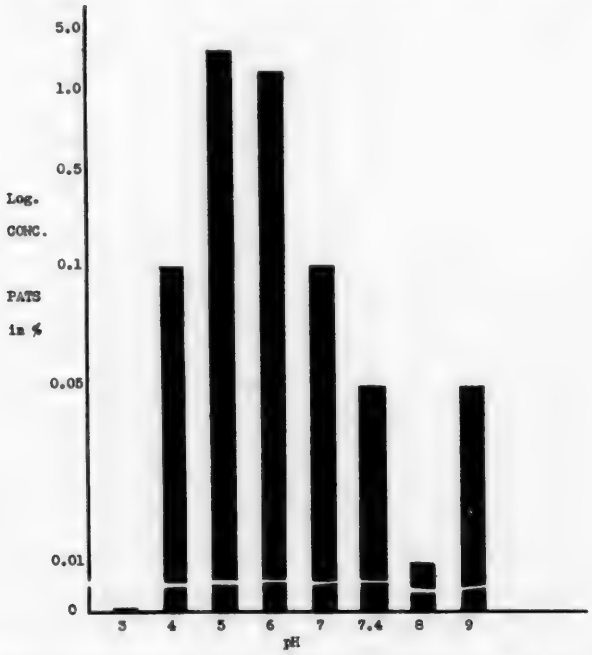

Fig. 1.—Maximum concentration of PATS showing bacterial growth in environments of differing pH (Culture 8).

TABLE I

BACTERICIDAL CONCENTRATION OF PATS AT OPTIMUM pH FOR THE RESPECTIVE CULTURES TESTED

CULTURE SAMPLE	GRAM STAIN AND MORPHOLOGY	MINIMUM BACTERICIDAL CONCENTRATION PATS (%)	OPTIMUM pH
1	Gram-positive cocci	0.10	7.4
2	Gram-negative and -positive cocci	0.01	7.4
3	Gram-negative diplococci	0.10	8
4	Gram-negative cocci	0.05	8
5	Gram-negative cocci	0.01	8
6	Gram-positive staphylococci	0.10	7.4, 8
7	Gram-positive staphylococci	0.10	7.4, 8, 9
8	Gram-negative cocci	0.05	7.4, 8
9	Gram-positive and -negative cocci	0.10	8, 9
10	Gram-negative cocci	0.05	8

evidence was regarded as indicative of bactericidal activity in the respective experimental solutions. Results typical of this portion of the project are shown in Fig. 2.

Since the bacterial cultures were maintained throughout the project, Cultures 7, 8, 9, and 10 were subjected a second time to the procedure described. The results of these tests were nearly identical with the initial ones for the respective cultures.

Toward the close of the project, it became obvious that the antibacterial activity of PATS probably bore some relation to the state of ionization of the molecule. To determine the relative state of the molecule, an electrometric titration was performed on the hydrochloride salt and, from the results so obtained, a titration curve was constructed as shown in Fig. 3.

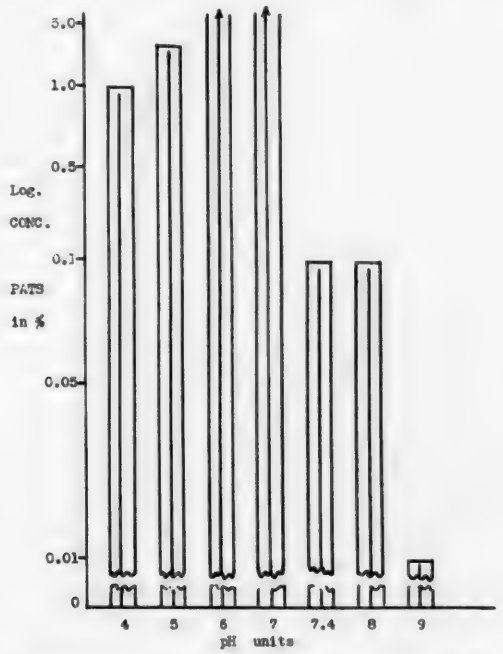

Fig. 2.—Minimum concentration of PATS showing bactericidal activity in environments of differing pH (Culture 8).

DISCUSSION

None of the samples of bacteria would grow in the experimental solutions at pH 3, although they grew nicely at pH 4. Subcultures made from these respective vials into plain brain heart infusion broth at its usual pH of 7.2, showed that the bacteria were nonviable. Evidently hydrogen ion from the acid medium is playing a role as bactericide. At pH 4, all of the bacterial cultures showed inhibition at a PATS concentration of 0.50 per cent or more; however, it seems logical that part of the antibacterial effect may have come from the hydrogen ion of the acid medium.

All bacterial cultures showed growth at higher concentrations of PATS when the environmental pH shifted from 4 to 5 (see Fig. 2). All but one of the ten cultures showed growth at the maximum PATS concentration when the environmental pH was 6 or 7. All but one of the ten cultures showed growth at markedly decreased concentrations of PATS when the pH was 7.4. All but one showed growth at the minimum experimental concentration of PATS when the solution pH was 8. Four of the cultures exhibited growth at an increased concentration of antiseptic when the pH was 9.

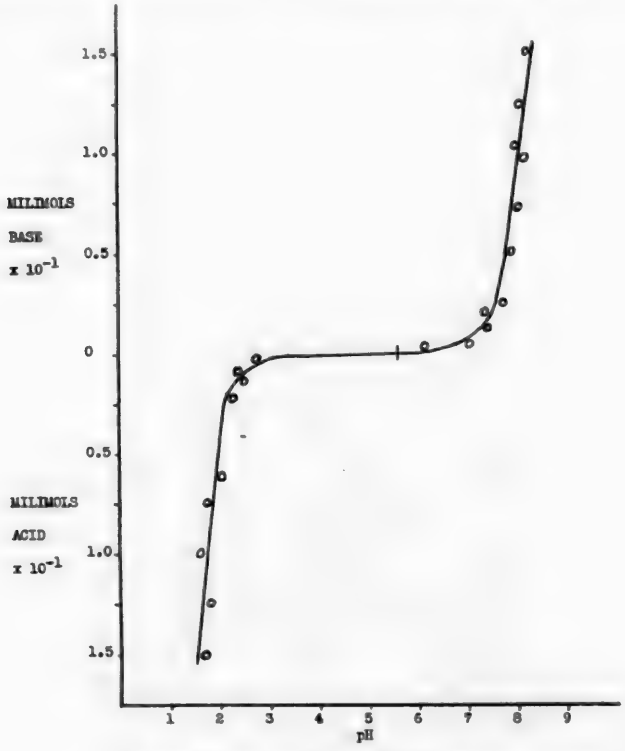

Fig. 3.—Titration curve of PATS hydrochloride.

It seems logical that the chemical nature of the PATS molecule plays an important role in determining the antibacterial activity of the drug. From the titration curve of Fig. 3, the probable nature of the PATS molecule can be determined.

Since the greatest antibacterial activity of PATS appears to be in an environment whose pH is between 7.4 and 8, let us consider that portion of the curve. The shape and behavior of the curve at this point indicates that the acid salt of PATS no longer exists, and that in its place we presumably have a nearly free base. It is this free base that exhibits the highest antibacterial activity, probably because of increased ability at penetration of

bacteria. This assumption, based on somewhat differing evidence, has been postulated previously by others in relation to the behavior of some of the common sulfonamides[2] and to the behavior of PATS.[4]

The bactericidal results shown in Fig. 2 are reasonably typical. In general, as the environmental pH rose from 4 to 7 the bactericidal concentration of PATS rose abruptly several thousandfold from a previously low value. At pH 7, nine of the ten cultures exhibited growth inhibition although no appreciable bactericidal action was evident at even the highest experimental concentrations of PATS. On the other hand, at pH 7.4 or 8, nine of the ten cultures exhibited bactericidal activity at PATS concentrations of 0.10 per cent or less (see Table I). The tenth culture exhibited maximum bactericidal action at pH 7, and drug concentration of 0.01 per cent.

In the bactericidal determinations, it is theoretically possible to introduce into the vials of plain brain heart infusion broth enough drug to inhibit or suppress bacterial growth. In a later paper, it will be shown that this possibility is nil for all practical purposes.

The relatively weak bactericidal activity of PATS in the pH range of 6 to 7 is probably associated with a predominantly uncharged molecular form. Since PATS is known to be an enzyme inhibitor,[5] it can readily be seen that alteration of its space charges could profoundly affect its activity.

From this study an acceptable bactericidal concentration of PATS at optimum pH would be in the region of 0.10 per cent. This value is somewhat higher than some values reported by others,[6, 7] but perhaps should be expected since endodontal bacterial mixtures generally require higher concentrations of drug to produce bactericidal action.

Howe, in his investigations on animals, used PATS buffered at pH 7.4, presumably because of the proximity of this value to that of tissue fluids.[3] Howe's results were highly satisfactory, a fact which might have been expected from this work. Waltner and White, using PATS for topical medication in otology, obtained the best clinical results when the PATS was at a pH of 2.2.[8] The implication arises that their clinical success was due in part to the antibacterial action of the acid medium.

SUMMARY

The antibacterial activity of PATS has been investigated in environments of differing pH. Under the experimental conditions described, the following factors become apparent.

1. As environmental pH changes from 4 to 6, the antibacterial activity of PATS decreases markedly.

2. At pH 6 to 7, there is marked lack of bactericidal activity, although bacteriostasis occurs at PATS concentrations of 3 per cent or less.

3. At pH 7.4 to 8, there is maximum bactericidal activity; the maximum bactericidal concentration being 0.10 per cent.

4. Maximum effectiveness of PATS appears to be associated with formation of the free base which presumably has greater penetrability and hence exhibits greater activity.

Although clinical situations cannot be compared to the conditions described, the implication is clear that the pH of a root canal, undergoing treatment with PATS, may play an important role in determining success of the treatment.

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A COMPARISON OF BONE GROWTH IN NORMAL AND STRONTIUM-TREATED RATS

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GROWTH of the skeletal system is not a uniformly progressive increase in the proportions of each member. The irregularities or complexities attending the normal differentiative growth of bones may be ascertained through histologic evidence, chemical data, or physical measurements. An attempt to formulate a possible interrelation among several of the physical changes associated with growth of the long bones of weanling animals was one of the aims of the present study.

Development of the tubular bones eventually resolves into two growth processes, transverse and longitudinal.[1] Because of the procedural simplicity, physical measurements of bones almost invariably include the measurement of length. A limitation of the increment in length is considered by some to be a satisfactory criterion of skeletal maturity. While the increase in length of a bone is an index of epiphyseal activity or longitudinal growth, the increment in volume of the bone reflects the degree of transverse growth as well. Little is known of the progressive increases in breaking strength or fracture resistance of bones during growth, and whether the strength of a bone increases in proportion to its length. A change in the breaking strength of the shaft of a tubular bone is probably determined primarily by periosteal and endosteal activity and regulated to an extent by function, among other factors. Elaboration of a technic for measuring the breaking strength of very small bones of young animals was an outcome of the present work.

Coupled with the study of normal bone growth was an attempt to determine the influence of strontium on the rapidly growing bones of albino rats. The stable strontium normally present in bone has been regarded as an innocuous trace element chemically similar to calcium. Current emphasis is on the physiologic differences between these two ubiquitous bone seeking minerals. Comparatively little attention has been focused on the study of possible toxic or abnormal effects upon developing bone resulting from the long-term ingestion of nonradioactive strontium. The present work deals with some of the long-range metabolic and physical effects of stable strontium on the bones of growing animals receiving an adequate diet. The over-all scope of the entire investigation embraces considerations of (a) lengthwise growth

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of a long bone, (b) increments in total volume of the intact bone, (c) increases in fracture resistance of the bone during growth, (d) changes in mineral content of the bone, and (e) uptake of strontium by the long bones.

MATERIALS AND METHODS

A total of 186 male weanling Osborne-Mendel rats of an inbred strain was apportioned among 5 groups. The pre-experimental group received the test diet and distilled water for one day, following which the animals were sacrificed. Two groups on a 90-day test period comprised 38 control rats and 39 rats receiving a strontium supplement. Another group of weanlings was placed on a 180-day strontium regimen while a companion group served as controls. Rats were housed in individual cages; food and water were available ad libitum. Controls were supplied with distilled water; both test groups received a 0.002 N solution of strontium nitrate (87.6 ppm strontium). A diet devised to supply the nutritional requirements without producing fluorosis had the following composition per kilogram: 250 grams casein, 100 grams Wesson oil,* 40 grams Hubbell-Mendel-Wakeman[2] salts,† 10 grams vitamin powder,‡ and 600 grams powdered sucrose.

This diet had a strontium content of 0.00074 per cent, or 7.4 ppm, determined by activation analysis.

After sacrifice, the rats were autoclaved for 10 minutes at 16 lb. in order to facilitate removal of soft tissue. The femora, humeri, and tibiae were then excised and placed in 10 per cent neutral formalin. Physical and chemical data were obtained by following the procedures described below.

a. *Length of the bones* was measured through the long axis, which was from the condyles to the greater trochanter in the case of the femur. The position of each bone in the micrometer was standardized, and measurements were read to 0.1 mm. with excellent precision.

b. *Total volume of the intact bone* is an index of peripheral enlargement. To obtain the *average cross-sectional area of a long bone* the volume of the bone (ml.) is divided by its length (cm.).

Volumetric readings can be estimated to within 0.001 ml. with a small test tube, a Koch microburet, and a meniscus reader. Test tubes whose inner diameter is just large enough to accommodate the selected bones are graduated at a level below which the bone will be fully submerged. The bone, which has been carefully cleaned and preserved in 10 per cent neutral formalin, is dried with cleansing tissue and placed in a calibrated tube. Neutral formalin from a microburet is introduced up to the graduation. The difference between the tube calibration and the volume of formalin introduced represents the total volume of the bone.

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*Containing 5,500 I.U. of vitamin A, 1,100 I.U. of vitamin D, and 200 mg. of α-tocopherol acetate.

†Excluding NaF, and including 100 mg. $CoCl_2$, 100 mg. $Na_2MoO_4 \cdot 2H_2O$, and 495 mg. $ZnSO_4 \cdot 7H_2O$ per kilogram of salts.

‡Containing the following in 1,000 Gm. of a mixture of lactose and vitamins:

400 mg. thiamin hydrochloride	10 grams niacin
250 mg. riboflavin	100 mg. vitamin K (menadione)
200 mg. pyridoxin hydrochloride	200 mg. folic acid
2,000 mg. calcium pantothenate	100 mg. biotin
100 grams choline chloride	10 mg. vitamin B_{12}

Air bubbles adhering to the bone and walls of the tube during filling are released by tapping the tube. Rigid control of the size of droplets delivered by the buret can be obtained by applying a water-repellent coating of silicone (Sight Savers) to the buret tip.[3] A clamp for holding the tube vertically consists of a meniscus reader* with a lead weight substituted for the magnifying lens. A smooth surfaced board serves as a horizontal platform for the clamp. A level is secured to the board, and adjustments are made by adding strips of tape to the underside of the platform. A one-inch hole in the board allows the tube to be slid up or down in the holder instead of drawing the buret up and down.

Some concept of the reproducibility of measurement using this volumetric procedure can be gained by noting the degree of variation in readings both with and without a bone in the graduated tube. The maximum deviation, based on 10 measurements in each case, was ± 0.002 ml. for both the calibrated tube (4.144 ml.) and the bone, an adult rat tibia (0.363 ml.).

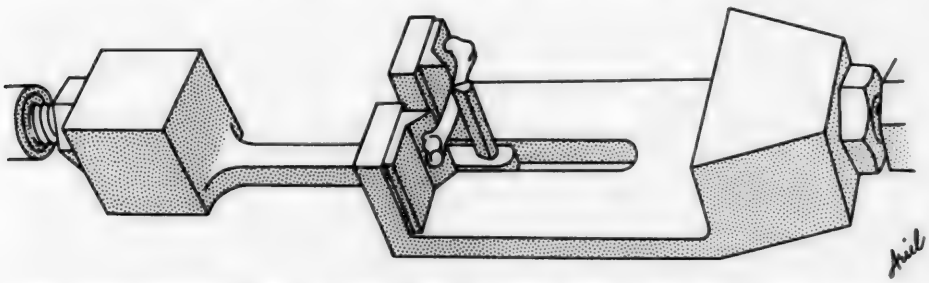

Fig. 1.—Specimen yoke used on incline plane materials tester. The paired knife edges are adjustable for testing segments of varying length.

c. *The relative breaking strength of wet bones* can be determined readily on a commercially available materials testing machine.† A specially designed yoke (Fig. 1) is substituted for the specimen clamps, enabling the machine to test the relative strengths of bones under static loading. The tester is based on the principle of the inclined plane, and the force is applied to the specimen at a constant rate. The failure load at the breaking point is determined from a chart on which the details of a test are traced by an autographic recorder while the test is being made.

The knife edges on the specimen yoke were designed to permit a variable span. A notch was hollow ground into one of the knives in order to securely seat a rat humerus. Mounted opposite the yoke is a fixed, central, opposing knife edge for grasping the bone at the midspan.

Excised bones are carefully cleaned and preserved in 10 per cent neutral formalin. Prior to testing, the length of the bone is measured and the midpoint marked. The wet bone is dried with cleansing tissue, placed in the tester, and the load is applied. After failure, the fragments can be retained for chemical analysis.

*Simplified model, A. H. Thomas Co., Philadelphia, Pa.
†Tensilgraph, Model IP-4, Scott Testers, Inc., Providence, R. I.

TABLE I
MEASUREMENT OF RELATIVE BREAKING STRENGTHS OF WEANLING AND ADULT RAT BONES*

AGE (MO.)	NO. OF RATS	BODY WT. (GM.)	BONE	SEGMENT TESTED (MM.)	RELATIVE BREAKING STRENGTH			
					LEFT BONE (LB.)	RIGHT BONE (LB.)	AVG. OF PAIR (LB.)	AVG. ABSOLUTE DIFF. BETWEEN LEFT AND RIGHT BONES OF A PAIR (LB.)
1	10	54 ± 7	Humerus	7	2.27 ± 0.46	2.26 ± 0.50	2.26 ± 0.47	0.12 ± 0.08
4	20	264 ± 42	Femur	14	22.73 ± 1.32	22.34 ± 1.48	22.54 ± 1.24	1.03 ± 0.86

*Values are expressed as the mean and the standard deviation. Bones were tested over a 7- or 14-mm. span and broke at the midpoint in each case.

Since there is no standard testing material with a predetermined strength in the desired range, and since a breaking strength test cannot be repeated on the same specimen, the error of measurement of any testing machine is only an estimate at best. An approximation of the precision of measurement of a machine can be made by testing the strengths of the right and left members of a pair of bones. Since there is no reason to assume that the two strengths must be identical, any lack of correspondence is not attributable entirely to the error of measurement. Table I summarizes measurements in two different ranges obtained with the tester described and indicates the reliability of the results obtained by this method. The material consisted of (a) weanling rat humeri with strengths of both right and left members ranging from 1.50 to 3.00 pounds, and (b) adult rat femora whose individual strengths ranged between 20.00 and 25.00 pounds. The specimens were bones of a consecutive number of tested rats in each case and included all rats with strengths in the specified ranges with no attempt to select pairs of bones with nearly identical strength. An average absolute deviation of 5.59 per cent for the weanling bones and 4.91 per cent for the adult femora is obtained by dividing the difference in strength between the two bones of a pair by the average strength of a pair.

The apparatus described above introduces several refinements over one used by the author previously[4] and permits the testing of very young bones as well. The procedure may also offer advantages over other technics,[5-8] since the method is convenient, very rapid, and dispenses with the use of water or lead shot as well as the need for drying the bones and embedding the ends in plaster of Paris. For broader aspects of biomechanics the reader is referred to the recent works of Bell[9] and Evans,[10] evidence of the growing interest in this field.

After demonstrating the dependability of the above-described static loading machine, the long bones of the 186 rats in the present experiment were tested in the following manner. The adult femora and humeri were fractured at their midpoints. Adult tibiae were broken at a point 7 mm. above the fibular notch. All adult bones were tested over a 14-mm. span. By reducing the span to 7 mm., midregions of the weanling femora and humeri were anatomically comparable to the adult bones. The tested segment of the weanling tibiae was 7 mm., the load being applied 3.5 mm. above the fibular notch. In order to approximate a comparison between strengths of young and adult bones, failure loads for the weanling bones were halved. Thus, all breaking strength values were based on a 14-mm. span. This oversimplification is in accordance with the formula *force* × *distance*.

d. *The mineral or ash content of the bones* was determined following the measurement of breaking strength. Each pair of dried bones was ashed to constant weight in a muffle furnace at 650° C.

e. *The strontium content of the ashed bones* was determined by neutron activation analysis. Bones were pulverized and passed through a 60-mesh screen, following which the powder was dried at 110° C. The humeri, tibiae

TABLE II
THE RELATION OF THE STRONTIUM CONTENT TO SOME PHYSICAL MEASUREMENTS OF THE LONG BONES OF RATS*

BONE	GROUP	NO. OF RATS	FINAL BODY WT. (GM.)	NO. OF DAYS ON EXPERIMENT	ANALYTICAL FINDINGS		PHYSICAL MEASUREMENTS		
					ASH WT. (MG.)	SR CONTENT OF ASHED BONE (%)	LENGTH (MM.)	TOTAL VOL. (ML.)	BREAKING STRENGTH (LB.)
Femur	Weanling	17	52 ± 7	0	33.2 ± 8.8	0.0242	18.9 ± 1.0	0.148 ± 0.023	1.14 ± 0.35
	Control	38	257 ± 52	90	295.8 ± 21.4	0.0130	32.3 ± 1.9	0.488 ± 0.061	22.03 ± 3.43
	Sr	39	265 ± 42	90	292.4 ± 38.8	0.2550	32.2 ± 1.3	0.485 ± 0.043	22.13 ± 2.90
	Control	43	348 ± 54	180	396.8 ± 63.6	0.0145	35.0 ± 1.7	0.571 ± 0.059	28.74 ± 4.43
	Sr	49	339 ± 60	180	401.2 ± 55.4	0.2350	35.3 ± 1.5	0.578 ± 0.049	29.40 ± 3.67
Humerus	Weanling	17	52 ± 7	0	21.3 ± 4.2	0.0215	16.4 ± 0.7	0.091 ± 0.013	1.06 ± 0.27
	Control	38	257 ± 52	90	126.9 ± 22.1	0.0141	25.3 ± 1.4	0.223 ± 0.024	12.11 ± 1.86
	Sr	39	265 ± 42	90	126.0 ± 16.7	0.2455	25.3 ± 0.9	0.222 ± 0.020	11.97 ± 1.63
	Control	43	348 ± 54	180	173.7 ± 26.6	0.0146	27.6 ± 1.2	0.257 ± 0.027	16.22 ± 2.28
	Sr	49	339 ± 60	180	174.8 ± 29.0	0.2270	27.8 ± 1.1	0.258 ± 0.022	15.70 ± 2.10
Tibia	Weanling	17	52 ± 7	0	32.0 ± 7.3	0.0227	22.5 ± 0.9	0.121 ± 0.016	0.92 ± 0.26
	Control	38	257 ± 52	90	242.9 ± 44.4	0.0120	35.5 ± 1.8	0.389 ± 0.053	17.06 ± 2.98
	Sr	39	265 ± 42	90	239.8 ± 31.2	0.2495	35.5 ± 1.3	0.386 ± 0.039	17.33 ± 2.37
	Control	43	348 ± 54	180	305.0 ± 47.7	0.0157	37.9 ± 1.6	0.446 ± 0.052	23.69 ± 3.53
	Sr	49	339 ± 60	180	308.6 ± 39.1	0.2050	38.2 ± 1.6	0.443 ± 0.037	23.36 ± 2.89

*Variation among animals is expressed in terms of the standard deviation. For the physical measurements the standard deviation is based on the mean value for the left and right bones of each animal.

and femora were each pooled by group making a total of 15 samples. Analyses were run in duplicate or triplicate on sample aliquots ranging in size from 0.0900 to 0.1110 gram. The values obtained showed an experimental spread in results which, in most cases, was within the ±5 per cent precision assigned to an activation analysis.

The actual determination was based on the neutron reaction, Sr^{86} (n, γ) $Sr.^{87m}$ $Strontium^{87m}$ has a half life of 168 minutes and emits a 0.388 mev gamma in its decay. After a neutron irradiation of at least 4 hours, each sample was processed by a radiochemical separation technic in which a known amount of inactive strontium was added to carry the radiostrontium through the procedure. Holdback carriers were added at various points in the separation so as to remove contaminant radioelements from the separated radiostrontium. Finally, the strontium carrier and radiostrontium were precipitated as the oxalate. The strontium oxalate was weighed to obtain the chemical yield of the carrier. The precipitate was then transferred to a suitable mount and the radioactivity measured in a gamma counter. The radiochemical purity of the precipitate was checked by means of a decay count and, in some instances, a gamma spectral analysis. The quantitative determination of strontium is based on the radioactivity of the unknown as compared with that produced in a series of samples containing known amounts of strontium which had been irradiated simultaneously with the unknowns and processed radiochemically in the same manner.

RESULTS AND DISCUSSION

The entire experimental data are condensed in Table II. Chronic toxicity in the treated animals was not apparent on the basis of body weight. The long bones of the treated rats showed no abnormality either in rates of longitudinal and transverse growth, or in gains in strength and mineral content. These results, based in part on relatively rapid phases of growth, confirm and extend previous findings showing the noneffect of stable strontium on the breaking strength and length of adult rat bones.[4]

The level of strontium in the weanling bones equalled that in ashed human bones (0.0234 per cent),[11] and this concentration was 1.5 times the per cent strontium in bones of control rats 180 days older. This reduction in initial strontium level reflects the relatively low strontium content of the diet and the ability of the young skeleton to eliminate or redistribute some of the strontium acquired before weaning. The per cent strontium in the bones of the treated rats was about 10 times that in the weanling bones. The absolute quantity of strontium increased between 90 and 180 days (Table III) during which time the femur, for example, increased in mineral content by 100 mg. However, the per cent or concentration of strontium in the long bones did not increase during this period (Table II). If the data suggest a metabolic state of strontium equilibrium, they do not indicate attainment of a saturation level, since much higher levels (5 to 6 per cent) of strontium in bone ash have been reported.[12]

TABLE III
THE ACCUMULATION OF STRONTIUM IN THE LONG BONES OF YOUNG AND ADULT ALBINO RATS*

GROUP	NO. OF DAYS ON EXPERIMENT	FEMUR	HUMERUS	TIBIA
Weanling	0	8.03	4.58	7.26
Control	90	38.45	17.89	29.15
Control	180	57.54	25.36	47.88
Strontium	90	745.62	309.33	598.30
Strontium	180	942.82	396.80	632.63

*Values are expressed in terms of micrograms of strontium per bone and are derived from the average ash weight of a bone and its strontium concentration in per cent.

The effect of age on dietary strontium uptake has not been clarified. A report[13] stating that a constant fraction (averaging 2.7 per cent) of the dietary strontium was found to accumulate in rat skeletons and that this degree of retention was not obviously influenced by the age of the rats appears to be inconsistent with the data in Table III showing an age effect on uptake of ingested strontium by the long bones. The discrepancy may lie in the fact that whole rat skeletons presumably include the continuously erupting incisors as well as the vertebral column in which the rate of mineral accretion exceeds that of all other skeletal components.[14] Thus, the total skeletal accumulation of strontium in *growing* adult rats may, within limits, appear to be proportional to the quantity of strontium ingested. However, age has been shown to appreciably influence the retention of ingested fluoride in the rat humerus,[14] and an analogous situation occurs in the case of strontium, as seen in Table III. The absolute quantity (micrograms) of strontium deposited in the long bones of rats consuming the strontium solution for 180 days was only 6 to 28 per cent greater than that acquired in the first 90 days on experiment. Long bones of the 180-day controls contained only 50 per cent more strontium than did the 90-day group. Per cent increase in strontium in all control bones between 90 and 180 days exceeded the per cent increase in ash for the same period, although the reverse was true during the first 90 days. Thus, the regulation of strontium uptake by factors other than the total mass of mineral matter is obvious.

While the various physical measurements (Table II) serve to compare test and control regimens, the results also pose questions as to the interrelations between mineral accretion, enlargement of external dimensions, and changes in strength of a bone. Table IV represents an attempt to analyze some of the complexities of the growth processes. Because of their remarkable agreement, values for test and control animals were combined. The tremendous increases in breaking strength of the bones 90 days postweaning is notable. An 1,800 per cent increase in the strength of the femur compared with a corresponding increase in length of only 70 per cent prompted a search for simple direct relations among the various factors. Expressing the per cent increase of any two factors as a ratio showed a consistency among the 3 bones, but the ratio was not constant for both age groups. Therefore, the per cent increments in length, strength, volume, and mineral content of a long bone seem to vary disproportionately with growth or maturation.

TABLE IV
SOME GROWTH CHARACTERISTICS OF THE LONG BONES OF THE RAT
PER CENT INCREMENTS FROM WEANING TO 4 MONTHS AND FROM 4 TO 7 MONTHS OF AGE

AGE PERIOD (MONTHS)	BONE	PER CENT INCREMENT				RATIO OF PER CENT INCREMENTS					
		LENGTH	TOTAL VOLUME	BREAKING STRENGTH	ASH WT.	ASH WT. TO LENGTH	ASH WT. TO VOL.	VOL. TO LENGTH	BREAKING STRENGTH TO ASH WT.	BREAKING STRENGTH TO VOL.	BREAKING STRENGTH TO LENGTH
1-4	Femur	70	228	1829	786	11.2	3.4	3.3	2.3	8.0	26.1
	Humerus	54	145	1136	493	9.1	3.4	2.7	2.3	7.8	21.0
	Tibia	58	220	1769	654	11.3	3.0	3.8	2.7	8.0	30.5
4-7	Femur	9	18	32	36	4.0	2.0	2.0	0.9	1.8	3.6
	Humerus	10	16	33	38	3.8	2.4	1.6	0.9	2.1	3.3
	Tibia	7	15	37	27	3.9	1.8	2.1	1.4	2.5	5.3

Percentagewise, the magnitude of transverse growth contrasted strikingly with that of longitudinal growth, which showed the lowest per cent gain of all the measurements. Transverse growth of the weanling femur in 3 months resulted in an increase in average cross-sectional area (vol./length) of 25 sq. mm., or an expansion of 325 per cent, compared with an increase in length of only 70 per cent. Yet, the lengthwise growth of the skeleton is an index of over-all bone growth, judging by our concept of "human growth in height, stature being quite an exact measure of skeletal size."[15]

Despite the varied patterns of fluctuation in per cent increase of the triad of physical measurements and ash weight, the two most sensitive of these variables, in terms of relative magnitude, are the breaking strength and ash weight. Breaking strength probably reflects localized changes associated with maturation as well as growth, being a measure of the bone's "utmost ability to resist force, i.e., to resist force to the point of fracture."[16] It is suggested that ash weights serve to ascertain the attainment of skeletal maturity in experiments with "adult" or "non-growing" rats. A termination of gain in skeletal ash seems to be a more sensitive, if not more dependable, criterion of skeletal maturity than the conventional termination of lengthwise growth of a tubular bone.

Formulation of an expression describing a relation between different measurements of bone growth was an aim of the present study. Table V is an approach to this objective. For all 3 tubular bones, a constant ratio of about 2 was derived by dividing the logarithms of the per cent increases in length, volume, strength, or ash for the first and second 90 days, respectively. This relation also applied to body weight, since the weight increases for the two periods were 402 per cent and 31.4 per cent, respectively, and log 402/log 31.4 = 1.74. The relation, however, does not hold for the logarithms of the absolute increases.

TABLE V
RELATIONS OF BONE GROWTH MEASUREMENTS IN THE RAT
RATIOS OF THE LOGARITHMS OF PER CENT OF INCREMENTS FOR FIRST AND SECOND 3-MONTH PERIODS AFTER WEANING

BONE	LENGTH	TOTAL VOLUME	BREAKING STRENGTH	ASH WT.
Femur	1.93*	1.88	2.17	1.86
Humerus	1.73	1.80	2.01	1.70
Tibia	2.09	1.99	2.07	1.97

*Values in this table are derived from Table III. For example, the increase in femoral length was 70 per cent in the first 3 months after weaning and 9 per cent in the succeeding 3 months. Thus, log 70/ log 9 = 1.93.

SUMMARY

The influence of a strontium supplement on bone growth was studied in male, Osborne-Mendel rats of an inbred strain over periods of 90 and 180 days postweaning.

1. Rates of longitudinal and transverse growth, gains in breaking strength and mineral component of the tubular bones showed no evidence of abnormality in the rats which had received strontium.

2. Both control and test animals showed a decidedly reduced rate of accumulation of ingested strontium in various long bones during 90 to 180 days postweaning.

3. The postweaning per cent gain in breaking strength of the tubular bones is remarkable, being 20 times the per cent increase in length after 90 days.

4. Growth in length, volume, strength, and ash of the long bones showed a twofold ratio between the logarithms of the respective per cent increases in these quantities for the first 3 months postweaning versus the succeeding 3 months.

5. Procedures are described for measuring the total volume of small animal bones with a precision averaging better than ± 0.002 ml. and for determining the relative breaking strengths of small bones with an average absolute deviation of less than 6 per cent.

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THE POLARIZED LIGHT APPEARANCES OF HUMAN DENTAL ENAMEL

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INTRODUCTION

SINCE the early studies of von Ebner,[3] linear polarized light has been used by numerous workers to investigate the microstructure of mature and developing human dental enamel.[1, 4, 5, 6, 8, 9, 11, 12, 13] In particular, the method has been used to study the distribution of the crystalline mineral matter. Briefly, the main conclusions reached by these investigators may be summarized as follows. Mature enamel, which is nonimbibable and shows negative intrinsic birefringence, consists of hydroxyapatite crystallites whose optic axes make angles of between 5 and 40 degrees with the long axes of the enamel prisms. Immature enamel consists of hydroxyapatite crystallites, similarly arranged to those in mature enamel, with intervening areas of organic matter containing imbibable spaces. This latter arrangement results, under suitable conditions, in the interaction of the negative intrinsic birefringence of the crystallites with the positive form birefringence of the spaces.

The following communication describes results obtained using, for the first time, an immersion medium of high refractive index, and also certain important anomalous features of the polarized light appearances of human dental enamel seen in numerous specimens.

MATERIAL AND METHODS

A formol-saline fixed upper permanent molar, with a clearly defined white hypomineralized area on its lingual surface, was selected from the routine hospital collection of extracted teeth to illustrate these anomalous appearances. From this tooth, a 30 μ buccolingual ground section involving the hypomineralized area was prepared.

A microradiograph of the section was taken on Kodak Maximum Resolution Plate using a Metro-Vickers Raymax x-ray unit operated at 30 kv., 15 Ma., copper target, nickle filter.

The following immersion media were prepared and their refractive indices checked on a Leitz-Jelley refractometer.

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1. Distilled water	n = 1.336
2. 70 per cent alcohol	n = 1.363
3. 90 per cent alcohol	n = 1.366
4. Absolute alcohol	n = 1.367
5. Absolute alcohol and xylol	n = 1.43
6. Xylol	n = 1.50
7. Methylene iodide and xylol	n = 1.569
8. Methylene iodide and xylol	n = 1.634
9. Methylene iodide and xylol	n = 1.686
10. Methylene iodide	n = 1 737
11. Methylene iodide and high refractive index fluid	n = 1.83
12. Methylene iodide and high refractive index fluid	n = 1.96
13. High refractive index fluid	n = 2.05

The high refractive index immersion fluid was that suggested by C. D. West.[14] It was prepared by gently warming eight parts by weight of phosphorus with one part by weight of methylene iodide and one part by weight of sulfur.

Starting with distilled water, the section was immersed for a total of one hour in each of the above fluids, except the last three in which the period of immersion was limited to 15 minutes because of inflammability. The appearance of the section was then noted in monochromatic polarized light—a sodium lamp being used as the light source. With a Berek compensator the retardations at various points were calculated for each fluid after 15 minutes, 30 minutes, and 60 minutes of immersion, each result being the average of two readings.

OBSERVATIONS

The microradiograph (Fig. 1) showed a central zone of low mineral content, within which there were mineral variations corresponding to the striae of Retzius. This central zone was surrounded by areas of well-mineralized enamel.

When the section was placed in distilled water and examined in linear polarized light (Fig. 2), the main mass of the enamel appeared negatively birefringent with a central positively birefringent area bordered by an isotropic zone. This positive area corresponded with the area of lowest mineral density seen on the microradiograph.

When immersed in absolute alcohol, after passage through 70 per cent and 90 per cent alcohol, positive zones bordered by isotropy slowly appeared on the occlusal, cervical, and surface aspects of the central positive area, which itself showed a slight decrease in positive birefringence (Fig. 3). The deeper parts of the surrounding well-mineralized enamel also showed a considerable reduction in the strength of their negative birefringence.

In absolute alcohol and xylol (n = 1.43), the central previously positive zone became completely isotropic, and a slight decrease was noted in the size of the peripheral positive and isotropic zones.

In xylol (n = 1.50), the central zone became weakly negatively birefringent, and the peripheral positive and isotropic zones showed further slight regressions.

In methylene iodide and xylol (n = 1.569), the central hypomineralized area became more strongly negatively birefringent, and the peripheral positive and isotropic zones showed a further slight regression and decrease in the strength of positive birefringence.

In methylene iodide and xylol (n = 1.634) (Fig. 4), the central zone showed a further increase in negative birefringence, and the peripheral positive and isotropic zones remained about the same. The deeper parts of the well-mineralized enamel were now slowly returning to the negative birefringence noted in water. At about this refractive index, the form birefringence should have been abolished, and thus theoretically the appearances should have represented only the intrinsic birefringence, and hence the distribution of the crystalline mineral matter.

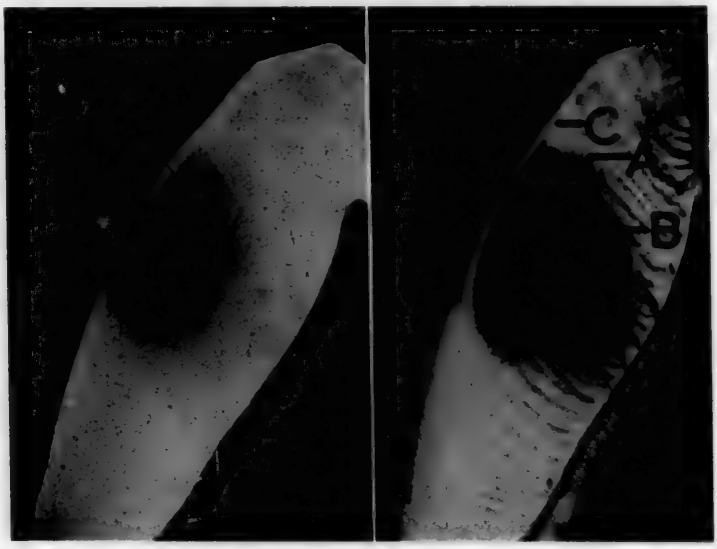

Fig. 1. Fig. 2.

Fig. 1.—Microradiograph of section showing central area of low mineral content. (×19.)
Fig. 2.—Same section as Fig. 1 showing negatively birefringent enamel with a central positive zone bordered by isotropy. Polarized white light. Immersed in water. (×19.)

The methylene iodide and xylol (n = 1.686) and methylene iodide (n = 1.737) media showed a decrease in the strength of negative birefringence in the hypomineralized central zone, but little other change.

On immersion in the three high refractive index fluids, the central zone became less negatively birefringent, isotropic, and, finally, more positively birefringent than in distilled water. The peripheral positive and isotropic zones showed a gradual enlargement (Fig. 5), and the deeper enamel showed a reducation in negative birefringence.

Three different types of behavior were thus exhibited by the enamel on passage of the specimen through the immersion media, and are represented

by the points *A*, *B*, and *C* (Fig. 2) at which retardation readings were taken. *A* was in the peripheral positive zone, *B* was in the central positive zone, and *C* was a surface area which showed minimal alteration during immersion.

A showed a behavior (Fig. 6, *A*) becoming positive in absolute alcohol and remaining so, which did not appear to be related to the refractive index of the immersion fluid. It was thus clear that the polarized light appearances of this area in all media, except possibly water, did not depend solely on the interaction of form and intrinsic birefringence as described for an ideal mixed body and, therefore, gave no accurate reflection of the amount of crystalline mineral matter present.

B showed the theoretically anticipated type of curve resulting from the interaction of positive form birefringence and negative intrinsic birefringence in an ideal mixed body (Fig. 6, *B*) and, in general, was compatible with existing theories on enamel microstruture.

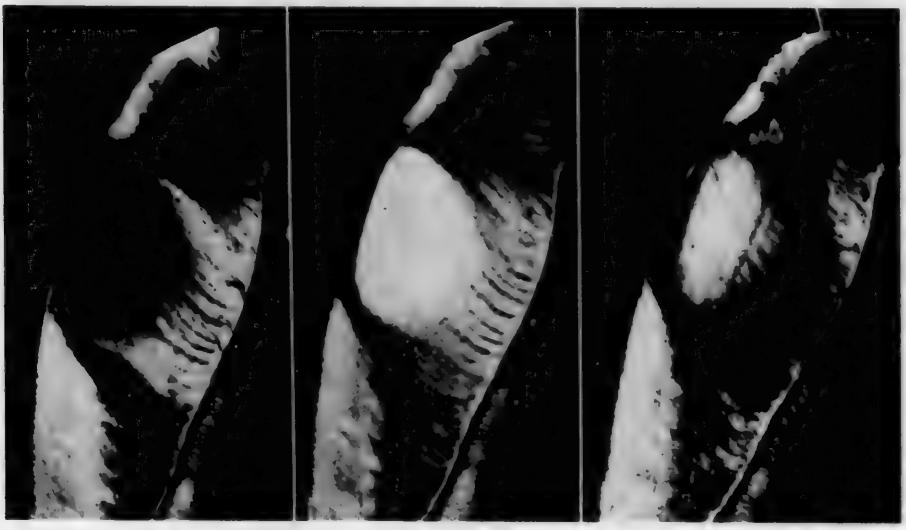

Fig. 3. Fig. 4. Fig. 5.

Fig. 3.—Same section as Fig. 2 showing slightly reduced central positive zone with surrounding positive and isotropic areas. Polarized white light. Immersed in absolute alcohol. (×19.)

Fig. 4.—Same section as Fig. 2 showing negatively birefringent central zone surrounded by positive and isotropic areas. Polarized white light. Immersed in methylene iodide and xylol (n = 1.634). (×19.)

Fig. 5.—Same section as Fig. 2 showing positively birefringent central zone surrounded by positive and isotropic areas. Polarized white light. Immersed in high refractive index fluid (n = 2.05). (×19.)

C showed very little alteration in negative birefringence, as anticipated in an area of well-mineralized, relatively nonimbibable enamel (Fig. 6, *C*). There was, however, a slight continuous decrease in negative birefringence between distilled water and the methylene iodide and xylol mixture (n = 1.686).

Comparing curves *A*, *B*, and *C* (Fig. 6), it further appeared that the negative intrinsic birefringence at *B* was greater than at either *A* or *C* in immersion

media of refractive index 1.569, 1.634, or 1.686, suggesting that *B* was better mineralized than either *A* or *C*. Reference to the microradiograph clearly showed this result to be completely erroneous, thus invalidating the distribution of mineral matter suggested by the polarized light appearances in these media.

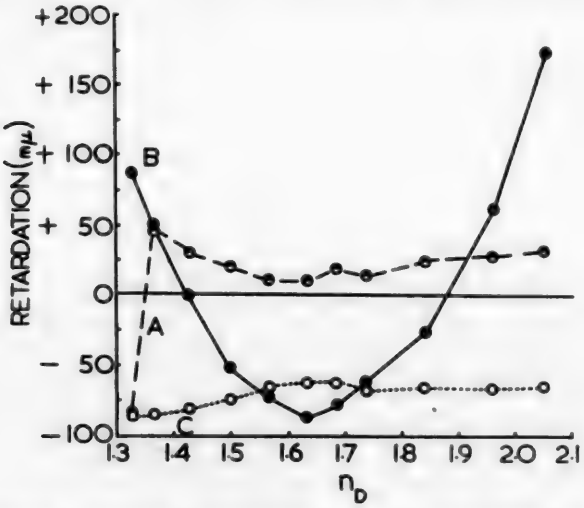

Fig. 6.—Effect of variation of the refractive index of the immersion fluid on the final retardation at points *A*, *B*, and *C*.

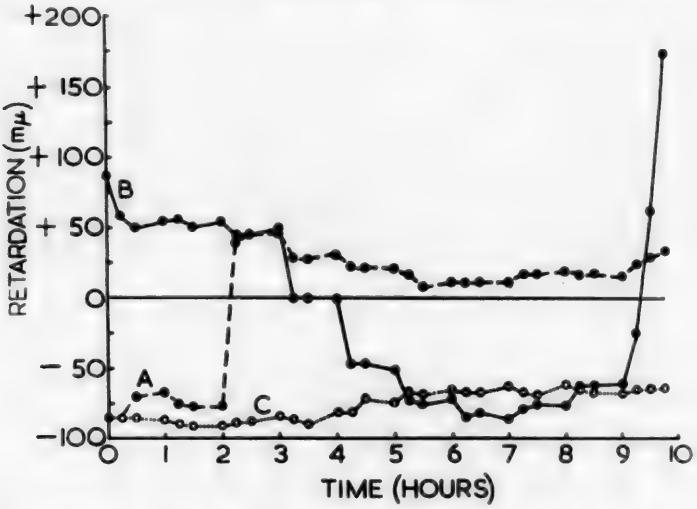

Fig. 7.—Effect of time on the retardation at points *A*, *B*, and *C* in the series of immersion fluids.

Little variation was noted in the retardation values obtained after 15 minutes, 30 minutes, and 60 minutes immersion in each fluid (Fig. 7). This result suggested that 15 minutes of immersion was as satisfactory as 60 under

the experimental conditions obtaining. Further experimental work has indicated, however, that immersion time can materially influence the polarized light appearance of the specimen.

DISCUSSION

These observations clearly indicate the operation of factors in addition to those considered when enamel is simply treated as an ideal mixed body of the rodlet type. Although the occurrence of such factors has previously been reported in the polarized light study of biologic materials, their interpretation is uncertain.

Schmidt[11] described two types of such additional factors. First, alterations in the strength and sign of the birefringence of keratin and collagen fibers produced either by temperature change, or immersion in acids or alkalis, or such media as water, ethyl alcohol, ether, formalin, glycerol, xylol, oil of cloves, cedar wood oil, monobromnaphthalene, and methylene iodide. These changes he interpreted as being due to the swelling of the immersed material, with disorientation of its miscellar structure resulting in alterations in its birefringence. Heat and immersion in concentrated solutions of potassium hydroxide, sodium hydroxide, carbolic acid, and potassium mercuric iodide were described as producing similar birefringence changes in elastin fibers.

Second, Schmidt described a birefringence change, von Ebner's phenol reaction, which occurs in collagen and elastin and is produced by the orientated adsorption of molecules of the immersion fluid on to the surface of the miscelles—a dichroic effect. This change was described as occurring with phenols and aldehydes, such as eugenol, aniline, cinnamon oil, and watery solutions of phenol and picric acid. An apparently similar phenomenon of the preferred orientation of embedding wax in decalcified sections of enamel organic matrix has recently been reported by Poole.[10]

Keil,[9] Darling,[2] and Schmidt[12] interpreted the anomalous birefringence changes in the outer parts of the caries lesion, as being due to the presence of gas or vapor-filled intermiscellar spaces. Keil considered the gas to be produced during the carious destruction of the enamel, while Darling considered it to be an artefact created during the withdrawal of water from the intermiscellar spaces by ethyl alcohol. Schmidt, in addition, suggested that the presence of the gas or vapor was the result of desiccation of the specimen with consequent shrinkage of the enamel organic matrix.

Considering the anomalous birefringence appearances reported in the present investigation in the light of these theories, it is clear that the disorientation of the miscellar structure would not account for the changes noted. It, therefore, seems most probable that the anomalous birefringence changes are produced by alterations in the properties of the imbibed fluid within the intermiscellar spaces, possibly of the type suggested by Schmidt[11, 12] or Darling.[2] Hartshorne and Stuart[7] have pointed out, however, that in fact the properties of the imbibed fluid are somewhat different from those of the free

liquid. It has a higher density, a lower vapor pressure, and a different refractive index. Moreover, there is the possibility, first shown by Maxwell, that the fluid may acquire an anisotropic character as a result of its various parts flowing with different velocities.

SUMMARY

The appearances of human dental enamel in linear polarized light are not completely explicable in terms of a rodlet type of Weiner ideal mixed body. A correlation of the microradiographic and polarized light findings, in the present investigation, suggests that the behavior of the immersion fluid within the intermiscellar spaces leads to anomalous birefringence appearances, which must be considered when estimating the degree of mineral density within different zones of the enamel. The results obtained when using, for the first time, an immersion medium of high refractive index (2.05) are also reported.

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THE EFFECT OF CASTRATION ON THE SUBMAXILLARY GLAND OF HUNT-HOPPERT CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS

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IN 1944, Hunt, Hoppert, and Erwin[1] reported the development of two strains of albino rats which differed from the common strains in their susceptibility to dental caries. When placed on a cariogenic diet, one strain showed a late and low incidence of caries; the other an abnormally early and high incidence. The caries-susceptible strain is difficult to maintain and shows signs of low vitality. Assays of the proteolytic enzyme secretion of the submaxillary gland have shown the protease activity of the susceptible animals to be 4 times that of the resistant strain.[2] It was found, however, that the proteolytic activity in the caries-susceptible strain was of the same order as in Sprague-Dawley rats[3] which are of intermediate caries susceptibility. The proteolytic activity in the caries-resistant strain, on the other hand, was comparatively low.

In Sprague-Dawley rats, the proteolytic activity of the submaxillary gland has been shown to be under partial control by the male sex hormone.[4] A similar control mechanism presumably exists in other strains of rats. The present study was undertaken to examine the role of the male sex hormone in male rats of the Hunt-Hoppert caries-susceptible and caries-resistant strains. The two strains were compared with respect to the time course of postnatal development and to the effects of castration.

METHODS

Controls.—Three developmental stages were studied—rats in early puberty (60 days of age), in late puberty (90 days of age), and young adult rats (150 days of age).

Castrate series: Littermates of the animals in the control group were castrated at approximately 30 days of age and sacrificed at 60, 90, or 150 days of age. Each group consisted of 4 to 9 animals.

The animals were killed with ether. The submaxillary gland was dissected, placed in liquid nitrogen and lyophilized in a freeze-drying unit (Waco). When dry, the glands were ground in a mortar and saline extracts were made. Protease activity was determined according to the method of

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Kunitz.[5] Saturating substrate conditions and optimal pH were determined in the group with the highest activity (150-day-old caries-susceptible animals) and kept standard for all other groups.

Assay Conditions

Substrate	1% casein
Gland extract conc. (dry wt.)	5 mg./c.c.
Incubation time	30 min.
pH	9.6
Temperature	37° C.

The results are expressed as units of optic density per milligram of nitrogen. Nitrogen determinations were made by the method of Ma and Zuazaga.[6]

FINDINGS

I. Caries-Susceptible Animals.—

A. *Controls:* The average specific proteolytic activity for Hunt-Hoppert and Sprague-Dawley rats at different ages is shown in Fig. 1 and Table I.

TABLE I. MEANS AND STANDARD DEVIATIONS OF PROTEASE ACTIVITY, O.D./MG.N, OF THE SUBMAXILLARY GLAND OF CONTROL AND CASTRATE HUNT-HOPPERT RATS

AGE (DAYS)	CARIES-SUSCEPTIBLE		CARIES-RESISTANT	
	NORMAL	CASTRATE	NORMAL	CASTRATE
60	0.13 ± 0.05	0.11 ± 0.02	0.09 ± 0.01	0.16 ± 0.01
90	0.45 ± 0.08	0.16 ± 0.06	0.11 ± 0.02	0.14 ± 0.03
150	0.56 ± 0.10	0.30 ± 0.06	0.23 ± 0.08	0.10 ± 0.03
	Significance: Normal vs. Castrate			
60	$0.5 > P > 0.4$		$P < .001$	
90	$P < .001$		$.025 > P > .01$	
150	$P < .001$		$P < .001$	

At 60 days, the proteolytic activity of the caries-susceptible strain was 0.13 unit. Between 60 and 90 days an increase of 3½ times occurred. At 150 days the activity was 0.56 unit.

In the submaxillary gland of rodents, increase in the size of the gland from puberty on occurs largely by an increase in the intralobular duct portion of the gland. The intralobular ducts are a secretory component and thought to be the site of proteolytic enzyme synthesis.[3]

At 60 days, most of the cells of the intralobular ducts of the caries-susceptible animals were cuboidal and had round, centrally placed nuclei. A few small granules were present in the cytoplasm. After 60 days of age, the cells underwent a transformation into secretory cells, in which the nucleus was flattened and pressed toward the periphery of the cell, and the cytoplasm became filled with coarse eosinophilic secretory granules. By 90 days, this differentiation was almost complete.

The same time course of postnatal development had been observed in Sprague-Dawley rats, in which the growth and secretory differentiation of the intralobular ducts and the specific proteolytic activity are accelerated after puberty is attained.[3]

B. *Effect of castration:* In the animals which had been castrated when they were 30 days old and sacrificed at 60 days, the proteolytic activity was about the same as in the controls. At 90 days, the proteolytic activity of the castrates was only a little higher than at 60 days (Table I and Fig. 2). It

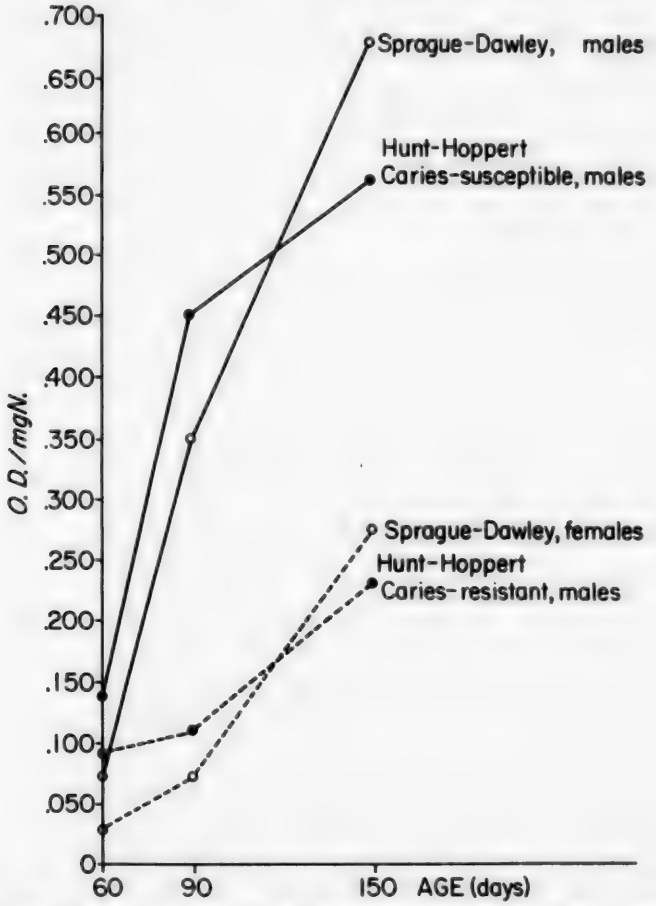

Fig. 1.—Mean proteolytic activity of the submaxillary gland of Hunt-Hoppert and Sprague-Dawley rats.

continued to increase at about the same rate until 150 days of age. Thus, at 60 days, the activity of the castrates was the same as in the controls; at 90 days it was only one third, and at 150 days about 55 per cent of that in the control animals.

At 60 days of age, the morphology of the submaxillary gland in the castrated animals was indistinguishable from that in the control animals. At 90 and at 150 days of age, however, the number of intralobular ducts per unit area was smaller than in the controls, the diameter of the ducts was less, there were few secretory granules in the cells, and the nuclei of the cells of these ducts were round and centrally placed.

II. Caries-Resistant Animals.—

A. *Controls:* The proteolytic activity in the caries-resistant strain did not show the postpuberal increase observed in the caries-susceptible and Sprague-Dawley animals. At 90 days, the activity was only a little higher than at 60 days. It doubled between 90 and 150 days. At 60 days, the proteolytic activity was a little lower than that of the caries-susceptible strain; at 90 days it was one fourth of it, and at 150 days less than half (Table I).

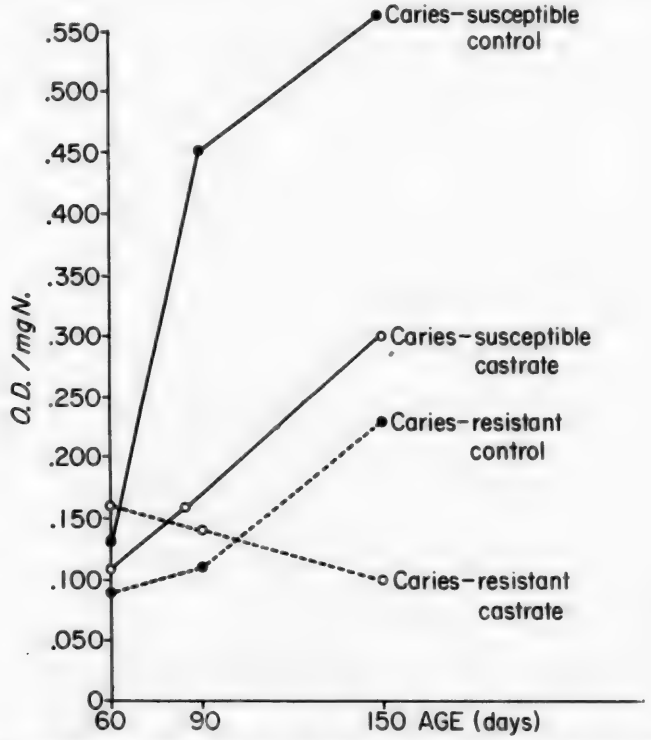

Fig. 2.—The effect of castration on the mean proteolytic activity of the submaxillary gland in caries-susceptible and caries-resistant rats.

The time curve of development was like that of the castrated caries-susceptible strain of rats (Fig. 2). This also resembled the time curve of development of normal female Sprague-Dawley rats (Fig. 1). The development of the intralobular duct portion of the submaxillary gland also failed to show the acceleration after puberty which characterized the development of the gland in the caries-susceptible strain and in the Sprague-Dawley males. Instead, it resembled that of castrated male caries-susceptible animals.

B. *Effect of castration:* Until 90 days of age, castration failed to lower the level of proteolytic activity below the control level. At 150 days, the activity was reduced to one half, as in the caries susceptible strain (Table I and Fig. 2).

The morphologic changes caused by castration proceeded in the same direction as in the caries-susceptible strain, but were much less severe.

III. Effect of Male Sex Hormone.—

Since the proteolytic activity of the submaxillary gland has been shown in Sprague-Dawley animals to be under partial control by the male sex hormone, a comparison of the absolute and relative weights of the testes of the Hunt-Hoppert 150-day-old caries-susceptible and caries-resistant rats was made. These are shown in Table II. The weight of the testis of the caries-susceptible animals is greater than that of the caries-resistant rats. The differences observed are significant to the 0.1 per cent level.

TABLE II. ABSOLUTE AND RELATIVE WEIGHTS OF THE TESTIS IN CARIES-SUSCEPTIBLE AND CARIES-RESISTANT RATS

	CARIES-SUSCEPTIBLE	CARIES-RESISTANT
No. of rats	13	9
Average body weight (Gm.)	308	342
Wet weight of testis (Gm.)	1.455 ± 0.133	1.223 ± 0.117
Relative weight × 10^3*	473 ± 32	359 ± 25

*Relative weight = organ weight/body weight.

SUMMARY AND CONCLUSIONS

Comparison of the postpuberal development of the submaxillary gland in caries-resistant and caries-susceptible Hunt-Hoppert rats shows that the caries-susceptible males resemble the Sprague-Dawley males. Both the developmental curve of specific proteolytic activity and morphology of the intralobular duct portion of the gland are similar in males of the caries-susceptible strain and the Sprague-Dawley strain. The caries-resistant strain remains underdeveloped in both respects. Castration interferes with the postpuberal development in the caries-susceptible strain as it does in the Sprague-Dawley animals, but has a less marked effect in the caries-resistant strain. Thus, the pattern of hormonal control appears alike in caries-susceptible and Sprague-Dawley animals, but is altered in caries-resistant animals. Both the absolute and the relative weight of the testis of control caries-susceptible rats is significantly greater than that of caries-resistant animals. The findings seem compatible with the hypothesis of an abnormally low androgen level in the caries-resistant males.

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THE EFFECT OF VITAMIN A DEFICIENCY UPON EXPERIMENTAL ORAL CARCINOGENESIS

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THE clinical and histologic changes seen in oral epithelium in both avitaminosis A and some precancerous conditions bear many similarities to one another. This study was initiated to investigate any possible relationship among these conditions.

Despite the fact that oral cancer, including cancer of the lip, constitutes 8 to 10 per cent of all cancer seen in the United States and is responsible for about 4 per cent of cancer deaths,[1] experimental oral carcinogenesis has only recently begun to receive attention. Levy,[2] in 1948, published a preliminary report of the effect of 20-methylcholanthrene on the labial gingiva of hamsters. Painting of this material for varying periods up to 10 weeks yielded negative results. In 1950, Levy and Ring[3] described methods for the implantation of crystals of 9,10-dimethyl-1,2-benzanthracene into the fundus of the hamster jaw. Sarcomas were produced in 80 per cent of the experimental group. Lutz, Fulton, Patt, Handler, and Stevens,[4] in 1951, reported success in the transplantation of methylcholanthrene-induced sarcomas to hamster cheek pouches. The first experimentally induced oral squamous-cell carcinomas were reported by Salley[5] in 1954.

Vitamin A deficiency has been primarily studied in the rat,[6] but epithelial changes have also been reported in the guinea pig,[7] fox,[8] mouse,[9] and monkey.[10] Recently, the hamster also has been subjected to vitamin A deficiency. Hirschi[11] studied bone healing in vitamin A deficient hamsters in 1950 and Salley and Bryson[12] described various tissue changes seen in the vitamin A deficient hamster in 1957.

The earliest recorded experimental production of neoplasms in animals fed a diet deficient in vitamin A was by Fibiger[13] in 1913. Fibiger produced tumors in the stomachs of rats fed bread and water using a parasitic worm which he named *Spiroptera neoplasticum*. In 1926, a Japanese investigator, Fujimaki[14] reported gastric papillomas when rats were fed a diet deficient in vitamin A. In 1936, Cramer[15] concluded that tumors could be produced by unbalanced diets, particularly those lacking in vitamin A, but that these were only contributory factors and not primary causes of the neoplasia. Rosicky and Halschek,[16] in 1944, reported that vitamin A had a retarding effect when it was added to benzpyrene and painted on the interscapular region of mice.

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Carminati and Baglioni,[17] late in 1955, investigated the relationship between the epithelial changes due to vitamin A deficiency, and subcutaneous carcinogenesis.

MATERIALS AND METHODS

One hundred and twenty golden hamsters *(Cricetus auratus)*, 25 days of age, were singly housed in an air-conditioned room. They were fed Nutrena Rabbit Chow for 20 days at the end of which period they were weighed and assigned to different groups. Each group contained an equal number of male and female hamsters of comparable weight. Table I indicates the total number of animals in each of the groups, their feeding regimen, and treatment. Water ad libitum was available to all animals.

TABLE I
EXPERIMENTAL DESIGN

GROUP	NUMBER OF ANIMALS	MALES	FEMALES	FEEDING REGIME	TREATMENT
I	40	20	20	(a) Vitamin A deficient diet (b) Ad libitum	0.5 per cent solution of 7,12-dimethyl-1,2-benzanthracene twice per week
II	30	15	15	(a) Vitamin A deficient diet (b) Ad libitum (c) Supplemented with 400 I. U. vitamin A per week	0.5 per cent solution of 7,12-dimethyl-1,2-benzathracene twice per week
III	30	15	15	(a) Vitamin A deficient diet (b) Pair-fed with Group I (c) Supplemented with 400 I. U. vitamin A per week	0.5 per cent solution of 7,12-dimethyl-1,2-benzanthracene twice per week
IV	10	5	5	(a) Vitamin A deficient diet (b) Ad libitum	Liquid petrolatum (solvent) twice per week
V	10	5	5	(a) Vitamin A deficient diet (b) Ad libitum (c) Supplemented with 400 I. U. vitamin A per week	Liquid petrolatum (solvent) twice per week

At 45 days of age, the hamsters were placed on an experimental vitamin A deficient diet. The diet was prepared to order in the form of bread and contained 6 ounces sugar, 4 ounces salt, 8 ounces lard, 4 ounces yeast, 1 gallon water, and 12 pounds flour. The flour was one third Pillsbury whole wheat flour and two thirds Pillsbury white flour.

All animals were fed the vitamin A deficient diet. Animals in Groups II, III, and V received, in addition, a weekly supplement of 400 I.U. of water-solubilized, natural vitamin A given orally. All groups were fed ad libitum with the exception of Group III which was pair-fed with Group I.

The carcinogen, 7,12-dimethyl-1,2-benzanthracene, was dissolved in liquid petrolatum heavy grade, U.S.P., to give a solution of 0.5 per cent concentration by weight. The carcinogen was topically applied to the left buccal cheek pouch with a cotton swab in Groups I, II, and III. Groups IV and V were treated in an identical manner applying only the solvent, liquid petrolatum.

The carcinogen was applied every Tuesday and Friday for 13 weeks. However, only 21 applications were given during this period for when more than one hamster showed marked suppurative reaction within the pouch the following application was not given. A waiting period of 7 weeks was allowed after applications ceased to permit latent changes to manifest themselves.

The animals were sacrificed by ether inhalation 20 weeks after they were started on the vitamin A deficient diet. The left buccal cheek pouch along with the submaxillary salivary gland, regional lymph nodes, and lung were dissected out and fixed in 10 per cent formalin. At the moment of death, cardiopuncture was performed and the blood of 5 to 8 animals from each group was drawn and pooled for a determination of the serum level of vitamin A by the Price-Carr antimony trichloride color reaction method. The cheek pouch and other organs were embedded in paraffin and sectioned at a thickness of 6 μ and stained with hematoxylin and eosin. The slides were "blinded" and examined microscopically.

OBSERVATIONS

After 5 weeks on the vitamin A deficient diet, symptoms of mild vitamin A deficiency began to appear in the unsupplemented groups when the fur of these hamsters lost its characteristic smoothness and became slightly ruffled in appearance. About the sixth or seventh week the weight gains began to level off. Fur changes became marked at 7 to 8 weeks. Very slight xerophthalmia became noticeable after about 9 weeks. From the eleventh week the animals began to lose weight.

TABLE II
AVERAGE WEIGHTS

GROUP	AVERAGE WEIGHT AT 45 DAYS OF AGE (GM.)	AVERAGE WEIGHT AT 195 DAYS OF AGE (GM.)	AVERAGE GAIN OR LOSS (GM. PER CENT)	
I	74.0	69.5	-6.0	-4.5
II	72.6	78.9	+8.7	+6.3
III	73.6	69.6	-5.4	-4.0
IV	80.0	81.1	+0.4	+0.3
V	74.3	86.9	+17.0	+12.6

The serum level of vitamin A in the animals receiving the supplement of vitamin A increased sharply shortly after administration and fell slowly until the next administration of the vitamin. At no time did the serum level in the

animals supplemented with vitamin A fall to the low level of the unsupplemented animals. Serum of the pooled blood of 5 to 8 vitamin A deficient animals (independent samples) was found to contain 19 μg per cent vitamin A. The serum of the pooled blood of 5 to 8 vitamin A supplemented animals varied in its vitamin A content from 57 μg per cent 5 hours after vitamin A administration to 26 μg per cent a week later when the next vitamin A administration was due.

The incidence of clinically observable tumors (Fig. 1) was greater in the unsupplemented group than in either of the other treated groups.

Fig. 1.—Hamster pouch, surgically opened, demonstrating multiple tumor masses.

On microscopic examination, the histologically prepared pouches were graded into 5 categories: (1) normal pouch epithelium (Fig. 2); vitamin A deficient epithelium (Fig. 3); (2) hyperkeratosis (Fig. 4); (3) benign papilloma (Figs. 5 and 6); (4) carcinoma in situ (Figs. 7 and 8); and (5) invasive squamous-cell carcinoma (Figs. 9 and 10). The incidence of malignant lesions in the unsupplemented animals was about 26 per cent greater than the supplemented ad libitum fed animals and about 34 per cent greater than those animals supplemented with vitamin A but kept on a paired-feeding regimen. Table III summarizes the histologic findings.

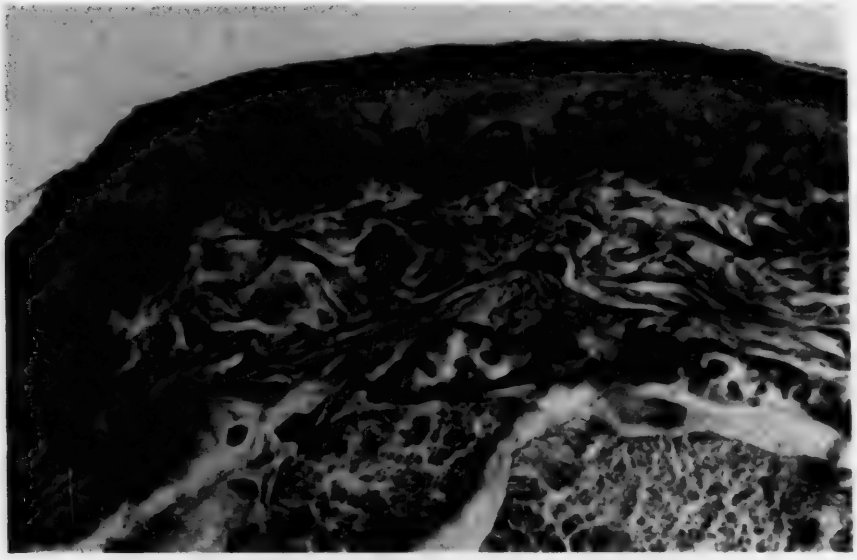

Fig. 2.—Normal cheek pouch epithelium of vitamin A supplemented hamster. (Orig. mag. ×415.)

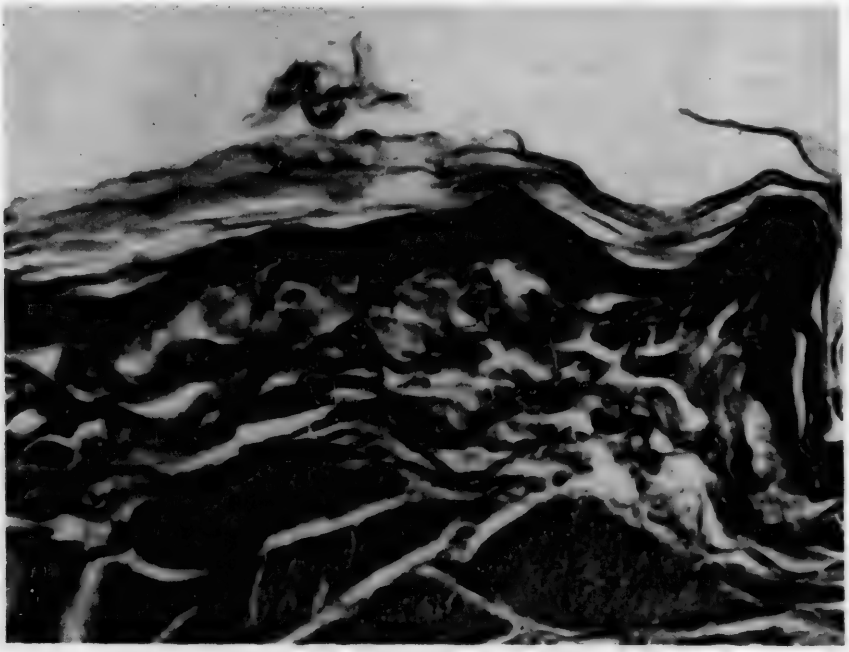

Fig. 3.—Atrophic, hyperkeratotic cheek pouch epithelium of vitamin A deficient hamster. (Orig. mag. ×415.)

TABLE III
TUMOR INCIDENCE AND HISTOLOGIC ANALYSIS

GROUP	NUMBER OF ANIMALS EXAMINED	NUMBER WITH CLINICALLY OBSERVABLE TUMORS	PER CENT CLINICAL TUMOR INCIDENCE	NORMAL EPITHELIUM	HYPERPLASTIC EPITHELIUM	BENIGN PAPILLOMA	CARCINOMA IN SITU	INVASIVE SQUAMOUS-CELL CARCINOMA	NUMBER WITH MALIGNANCIES	PER CENT INCIDENCE OF MALIGNANCIES
I	27	22	81.5	1	3	7	14	2	16	59
II	27	20	74.0	5	4	9	7	2	9	33
III	12	7	58.3	1	2	6	3	0	3	25
IV	6	0	0	6	0	0	0	0	0	0
V	8	0	0	8	0	0	0	0	0	0

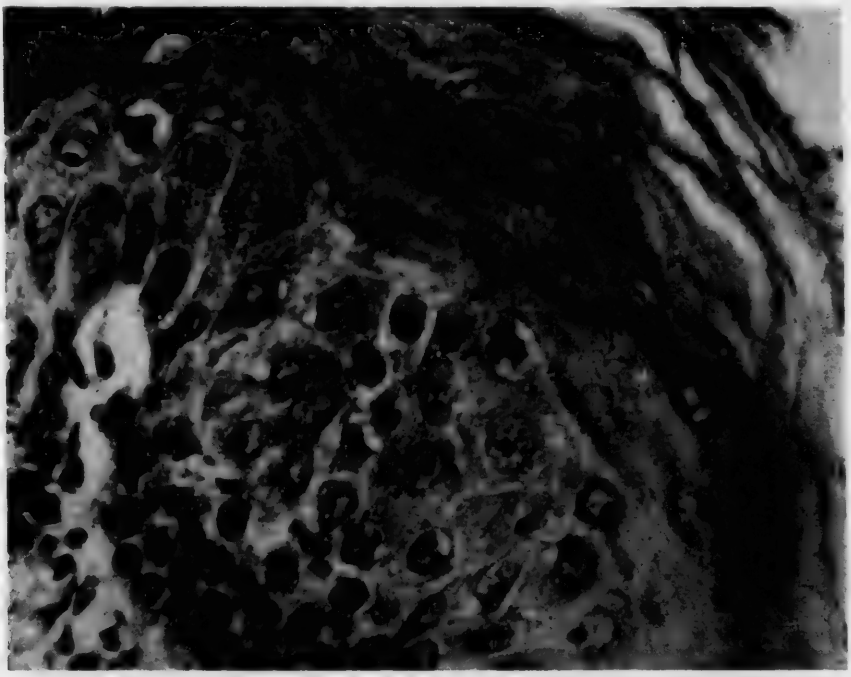

Fig. 4.—Hyperplastic epithelium cheek pouch of hamster. (Orig. mag. ×415.)

DISCUSSION

Vitamin A deficiency in the rat can be readily produced experimentally. However, attempts in this laboratory to produce oral carcinogenesis in this animal have not been successful. The hamster, because of its unique pouch, in which cancer has been successfully induced by several investigators,[5, 18] was selected as the more suitable animal. The hamster is also subject to vitamin A deficiency and therefore fulfilled both requirements for this investigation.

Fig. 5.

Fig. 6.

Fig. 5.—Cheek pouch epithelium exhibiting benign papilloma. (Orig. mag. ×22.)
Fig. 6.—Benign papilloma. (Orig. mag. ×125.)

Fig. 7.

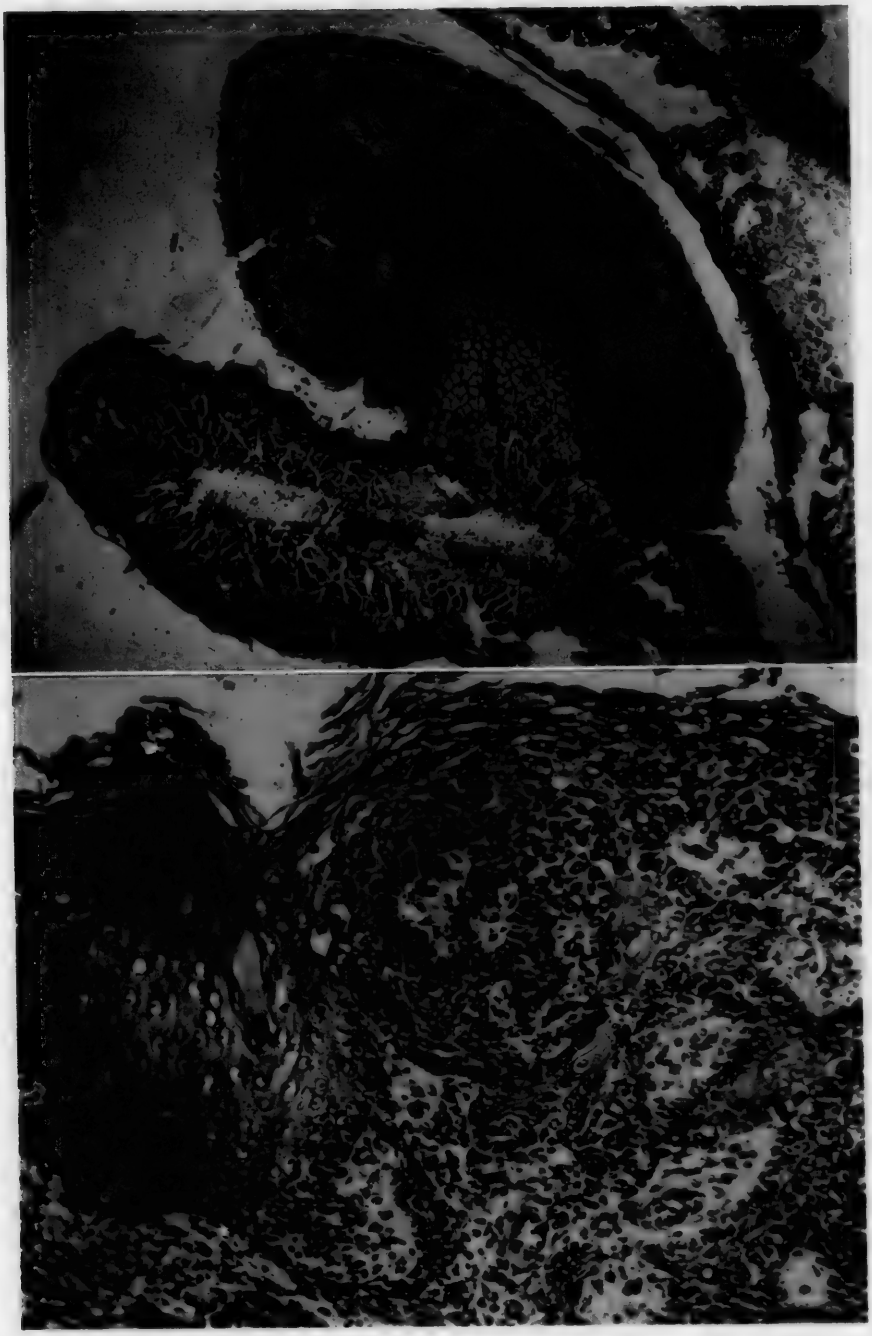

Fig. 8.

Fig. 7.—Cheek pouch epithelium exhibiting carcinoma in situ. (Orig. mag. ×22.)
Fig. 8.—Carcinoma in situ. (Orig. mag. ×125.)

Fig. 9.

Fig. 10.

Fig. 9.—Cheek pouch epithelium exhibiting invasive squamous-cell carcinoma. (Orig. mag. ×22.)

Fig. 10.—Invasive squamous-cell carcinoma. (Orig. mag. ×415.)

Producing the two conditions simultaneously presents difficulty. The production of deficiency symptoms appears relatively early while neoplasia appears only after extensive periods. Animals deficient in vitamin A are subject to numerous intercurrent diseases and therefore are likely to die before tumors are visible. Therefore, a carcinogen with potency sufficient to produce tumors in a relatively short time must be used. Furthermore, hamsters do not survive when fed vitamin A test diet (U.S.P.) for the rat. A special vitamin A deficient diet able to supply all other nutritive requirements of the hamster had to be prepared in order that the animals would live sufficiently long to exhibit neoplasia.

Since animals fed a vitamin A deficient diet often lost their appetite, their caloric intake was thus reduced. Tannenbaum[19] has established that a diet with a restricted caloric content plays a definite inhibitory role in the development of tumors. In order that we might compare vitamin A deficient animals with animals fed identically except for the vitamin A supplement and not animals with restricted caloric intake with normal animals, a third group to be pair-fed with the vitamin A deficient group was added.

Vitamin A deficiency produces metaplastic changes in the epithelial linings of the trachea and bronchi which predispose the animal to pneumonia and other respiratory disorders. The observed death rates reflect this fact, the fatality rate of the vitamin A deficient animals being two to three times as high as for animals supplemented with vitamin A weekly.

While the average weight loss of the various groups may in part have been related to the group death rate, other factors undoubtedly exist. This inference is substantiated by the fact that while Group I had a death rate of 27.5 per cent and an average weight loss of 6 per cent, Group III had a death rate which exceeded that of Group I by more than twice the value and yet had a smaller average weight loss.

Vitamin A determinations were made on serum rather than on liver because several investigators[20-23] have demonstrated that vitamin A may be absent from the liver while still present in serum and other sites. The samples of blood, drawn when animals were sacrificed, demonstrated that even though the animals on the vitamin A deficient diet had received no vitamin A for 20 weeks, the serum still contained a low level of the vitamin. This is in agreement with the work of these investigators who have shown that the serum vitamin A level falls very slowly until total depletion of the labile body stores has been achieved, death occurring shortly thereafter. Thus, much as it is desirable to compare totally depleted animals with normal animals, in a study of this duration, this was not possible. The serum determinations showed that the serum vitamin A level of the vitamin A deficient group was lower than that of the serum of the vitamin A supplemented animals. The results, then, compare 2 groups of animals where vitamin A is present in the serum at two different levels rather than two groups in which vitamin A is present in one and absent in the other. The data obtained from this investigation must be evaluated in this light.

A comparison of Group I with Group III would be the most logical method of analyzing the effect of vitamin A deficiency upon carcinogenesis without clouding the results by dissimilar caloric intake. Neoplastic changes were observed more frequently both grossly and microscopically in the vitamin A deficient group. In both Groups II and III (receiving oral vitamin A supplements) a greater number of animals exhibited either no epithelial change or changes within the range of benign hyperplasia. In contrast, the vitamin A deficient animals demonstrated epithelial changes suggestive of various degrees of malignancy.

A statistical evaluation of the histologic data indicated that the probability that the numerical difference between Group I and Group III would be exceeded by chance error alone is less than 5 per cent. We may conclude from the data obtained that a deficiency of vitamin A in the hamster may promote malignant changes in the epithelium of the cheek pouch when a carcinogen is applied.

SUMMARY AND CONCLUSIONS

This study indicates that:

1. In the carcinogen-treated cheek pouch of the golden Syrian hamster, vitamin A deficiency promotes epithelial tumor production.

2. A higher tumor incidence in the group fed ad libitum than in the group with restricted caloric intake is in agreement with the findings of Tannenbaum, namely, that tumor production is inhibited by a diet with restricted caloric intake. However, this effect appears to be more than offset if the diet is deficient in vitamin A.

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HALF THICKNESS OF DENTIN TO P_{32} BETA RADIATION

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INTRODUCTION

IN previous research, the authors have made use of P_{32} as a tracer in the evaluation of various cavity liners. However, the radioautographs have proved to be inconclusive due to the diffuse shadow of the isotope. It was not possible to detect whether the isotope had or had not penetrated the liner, because there was no means of differentiating the shadow made by the penetration of tracer from that made by the radiation coming through dentin from tracer which had not penetrated. For the solution of this problem, it is necessary to determine how far radiation can pass through dentin. A search of the literature revealed no investigation in this area. Thus, the purpose of this study was to determine (a) the relationship between dentin thickness and the relative intensity of radiation, and (b) the half thickness of dentin to beta radiation of P_{32}. Such data are pertinent to interpretation of radioautographs made with this isotope.

PROCEDURE

Extracted human upper central incisors were used. Forty dry teeth, stored for one to 3 years after extraction, were cut into small dentin plates approximately one millimeter thick. Half of these specimens were sectioned parallel and half perpendicular to the dentinal tubules, as shown in Fig. 1. In a second group, 10 teeth were immersed in a physiologic sodium chloride solution immediately after extraction, and dentin sections perpendicular to the dentinal tubules were prepared. These sections were made at time intervals ranging from one to 7 days after extraction.

The Lauritzen electroscope (Fig. 2) was used for measuring the intensity of radiation. A piece of filter paper previously soaked in P_{32} (as the phosphate) solution and dried thoroughly in air was used as a beta source. Specimens were placed on a lead diaphragm having a small round hole approximately 0.5 mm. in diameter. The time required for the electroscope fiber to move over a given scale division by the ionic discharge due to the radiation was measured.

Three consecutive readings were made on each specimen, each time the thickness was reduced by grinding. The exact thickness of each dentin specimen was determined by a micrometer.

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RESULTS

The results can be seen in Figs. 3, 4, and 5. The intensity of radiation is inversely related to the discharging time of the Lauritzen electroscope[1] and varies approximately in direct logarithmic proportion to the thickness of the absorber[2] (the dentin specimens). Consequently, the relative intensity was calculated as a percentage of the ratio of the reciprocal of time readings to that of the reading made with no specimen in place. This figure was plotted logarithmically on the vertical axis of the graphs.

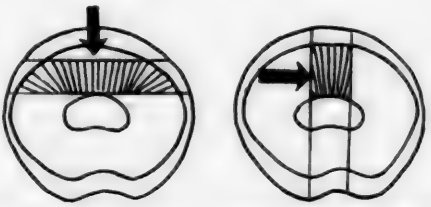

Fig. 1.—Directions of section and radiation to dentinal tubules.

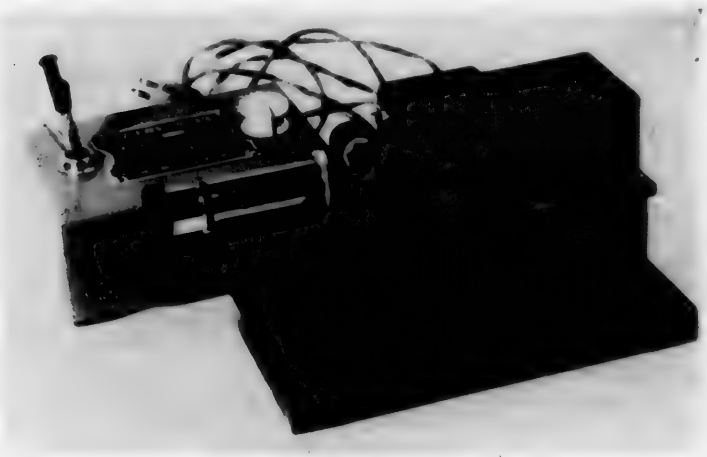

Fig. 2.—Lauritzen electroscope.

$$\text{Relative intensity} = \frac{\text{discharging time without specimen}}{\text{discharging time with specimen}} \times 100.$$

From these curves, it is apparent that approximately 0.4 mm. of dentin thickness is needed to halve the radiation intensity and more than 1.0 mm. is needed to cut it down to one third.

The so-called half thickness of dentin to P_{32} beta rays was computed in the following manner. The curves for individual specimens were made by connecting 3 points on the grafts, representing 3 measurements of each specimen. The half thickness of individual specimens, i.e., the thickness halving the intensity of radiation, was determined on those individual curves.

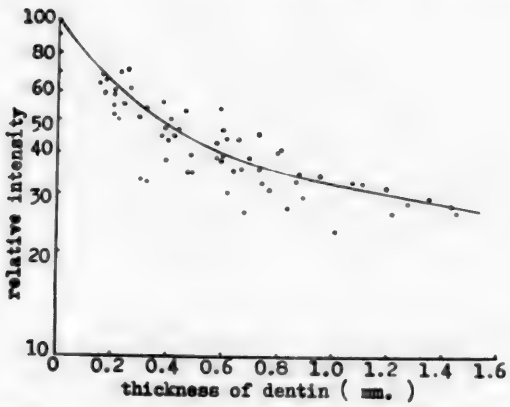

Fig. 3.—Relationship between the absorbing thickness of old dry dentin and the P_{32} radiation, parallel to dentinal tubules.

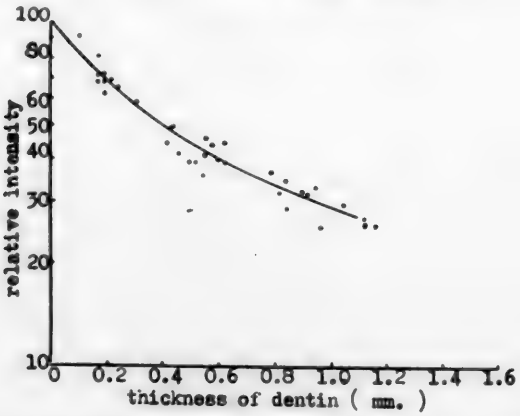

Fig. 4.—Relationship between the absorbing thickness of old dry dentin and the P_{32} radiation, perpendicular to dentinal tubules.

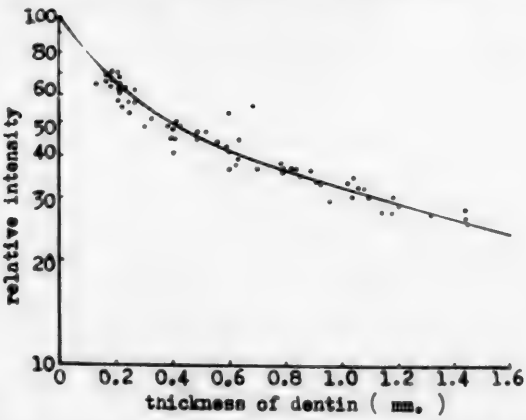

Fig. 5.—Relationship between the absorbing thickness of fresh wet dentin and the P_{32} radiation, parallel to dentinal tubules.

The results are summarized in Table I. The deviation of the results is shown with standard deviations for simplification, although the distribution was not of normal curve. It will be noted that the deviations were rather small.

TABLE I
HALF THICKNESS OF DENTIN TO P_{32} BETA RAYS

DENTIN PLATE	DIRECTION OF RADIATION	HALF THICKNESS
Old and dry	Parallel to dentinal tubule	0.36 ± 0.11 mm.
Old and dry	Perpendicular to dentinal tubule	0.37 ± 0.08 mm.
Fresh and wet	Parallel to dentinal tubule	0.39 ± 0.04 mm.
Summary of three groups		0.38 ± 0.09 mm.

The differences between the 3 groups were not statistically significant. Thus, no difference for half thickness was found between specimens prepared so that the beta rays passed parallel to the dentinal tubules and those sectioned so that the rays passed perpendicular to the tubules. Likewise, no difference was detected between old, dry dentin and comparatively fresh, wet dentin.

The average for the half thickness of the 3 groups was 0.38 ± 0.09 mm. This result indicates that a marked beta radiation can pass through dentin of a thickness of 0.4 mm. or greater. The radiation produces a diffusive shadow on the autograph without any actual penetration of the isotope itself. This would seem to indicate that detection, by radioautographs, of a tracer penetration of a rather short distance and through a wide area such as would be employed in studying cavity liners, by use of a P_{32} beta source, would be most difficult.

CONCLUSION

The relationship between the thickness of human dentin and the relative intensity of P_{32} beta rays, after passing through dentin, were studied by means of a Lauritzen electroscope.

The half thickness of human dentin to P_{32} beta radiation was 0.38 ± 0.09 mm. The direction of radiation, in relationship to the dentinal tubules, and the dryness or age of the dentin specimen had little effect on the half thickness.

The use of this tracer in the evaluation of cavity liners by radioautographs would seem to be contraindicated.

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ANTIBACTERIAL ACTION OF CERTAIN FLUORIDE-CONTAINING DENTAL RESTORATIVE MATERIALS

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THERE is increasing evidence that none of the currently used restorative materials actually seals the cavity preparation.[1-4] Factors such as temperature change,[5] galvanic action,[6] and solubility of certain materials[7] further enhance the possibility of either minute or gross leakage. Recognition of these problems has renewed interest in the antibacterial properties of certain restorative materials. Differences in bacteriologic technics, types of microorganism and culture media used, and variability in transfer of platings have resulted in a lack of conclusive evidence regarding the exact effectiveness of each material.[8-12]

It is generally recognized that the incidence of recurrent caries around the silicate restoration is markedly less than with other types of restorative materials.[13-15] It has been suggested that this unique characteristic can be attributed to a reaction of the fluoride in the silicate with the surrounding enamel and dentin to reduce its solubility, rather than the fluoride acting as a germicidal agent or enzyme inhibitor.[7, 14] It has also been shown that low concentrations might be added to inert materials, such as resins, to provide a similar effect. The purpose of the investigation was to determine whether the added fluoride might also provide certain bacteriostatic properties.

PROCEDURE

A list of the commercial and experimental materials studied can be seen in Table I.

Media and Organisms.—The types of media chosen were those considered to be most favorable for the growth of each test strain.[16-18] Sterile horse serum or sterile human saliva was added to the media in order to make the experimental conditions somewhat more comparable to those of the oral cavity.

1. Lactobacillus—selection medium (L. B. S.) was used for *Lactobacillus acidophilus* var. *odontolyticus* and *Lactobacillus casei.*

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TABLE I

Silicates	
	S. S. White Filling Porcelain—S. S. White Co.
	DeTrey's Filling Porcelain—L. D. Caulk Co.
Resins	
	FluorOn—American Consolidated Mfg. Co.
	*Experimental Resin + 0.05% SnF_2
	Experimental Resin + 0.50% SnF_2
	Experimental Resin + 2.0% SnF_2
	Experimental Resin + 0.05% NaF
	Experimental Resin + 0.50% NaF
	Experimental Resin + 2.0% NaF
	Experimental Resin + 5.0% NaF
Zinc Phosphate Cements	
	S. S. White Zinc Cement—S. S. White Co.
	*Zinc Phosphate Cement + 5% CaF_2

*Experimental materials prepared by courtesy of L. D. Caulk Co., Milford, Del.

2. Trypticase soy agar was used for *Streptococcus viridans, Staphylococcus pyogenes* var. *aureus, Staphylococcus pyogenes* var. *albus* and *Escherichia coli.*

3. Brewer's anaerobic medium was chosen for *Clostridium sporogenes.*

The horse serum and saliva were added to these media in a concentration of 0.3 ml. per 30 ml. of the melted medium.

Aseptic procedures were utilized as much as humanly possible. All instruments used in the experiments were sterilized by autoclaving under 15 pounds pressure for 20 minutes at 121° C. Saliva was stimulated by chewing gum and collected before breakfast. It was sterilized by filtration through a Seitz filter.

Media used in these experiments were prepared and sterilized according to the manufacturer's directions. Thirty milliliters of the medium were melted, cooled to approximately 47° C., and inoculated with 0.3 ml. of a saline suspension of the test organism. The same quantity of medium poured in a Petri dish was used in each test. The sterile horse serum or human saliva was added prior to inoculation with the test organism. The cement was mixed to a consistency thick enough to be pushed off the spatula and, as soon as the mix was completed, it was transferred to the Petri dish. In order to keep the pellet size as uniform as possible, a steel ring with an inside diameter of 11.1 mm. was used as a guide. The ring was placed under the plate and the cement manipulated until the diameter of the pellet coincided with that of the ring underneath. After the medium was firmly set, the plates were incubated at 37.5° C. in an inverted position for 48 hours, with the exception of those plates inoculated with *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes.* These were incubated for 72 hours. When *Cl. sporogenes* was used as the test organism, the plates were placed in anaerobic jars and stored in the incubator.

At the end of the incubation period, each plate was examined for a zone of growth inhibition. A bacterial plate counter was used and the width of the zone was measured by a millimeter caliper from the edge of the pellet to the periphery of the zone of inhibition.

In order to study the "exhaustion" (the state at which the material fails to inhibit bacterial growth) of the antibacterial effect of the material, the pellet was transferred with sterile forceps to another freshly inoculated plate. This plate was incubated as previously and subsequently examined for a zone of inhibition. Pellets were transferred to new plates only when there were zones of inhibition in the original plates.

Two additional series of tests were run under the following set of conditions:

a. Inoculated plates were placed in a refrigerator at 9° C. for 12 hours. They were then incubated for 48 hours at 37.5° C. and examined for zones of inhibition. However, plates inoculated with *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes* were not examined until the end of 72 hours. The purpose of this procedure was to observe if any readily soluble inhibitory material leached out of the specimens before active growth of the microorganisms, thus resulting in an increased zone of inhibition.

b. In order to discover if any slightly soluble inhibitory material would leach out after the first 48 hours (incubation), the plates were held at 37.5° C. for 168 hours and then examined.

Experiments With Set Pellets.—The same procedure was followed in this series of experiments except the cement pellets were permitted to harden 18 to 24 hours before being placed on the agar plates. It was felt that experiments with the hardened pellets would detect whether any soluble material leached out and inhibited bacterial growth after the cement had set.

Experiments With Cement Powders and Liquids.—In order to observe the action of the separate constituents of the cements, tests were made using the individual powders and liquids. In these tests no horse serum or human saliva was added to the media used. Plates were poured and a measured amount of cement powder transferred from the spatula and pressed into the semiset medium. In those experiments involving the liquids, one drop was placed on the surface of the inoculated, solidified medium. All plates were incubated at 37.5° C. for 48 or 72 hours and then examined for zones of inhibition.

RESULTS

Results on plastic pellets for only 6 of the cements are shown in Tables II-VII. These data are representative of each class of material. Each figure is the mean of results obtained with a minimum of 3 test specimens. In Table VIII can be seen the data obtained with the various cement liquids. Since the results with the powders, the hardened specimens and pellets transferred to freshly inoculated plates were negative, none of these data are shown.

Plastic Pellets.—One silicate exhibited antibacterial action against *Staph. pyogenes* var. *aureus, Staph. pyogenes* var. *albus, Str. viridans,* and *Esch. coli* but was ineffective when tested against *L. casei* and *Cl. sporogenes* (Table II). The resin cements, even with high concentrations of fluoride, had no effect against any test organism other than *Esch. coli* (Tables III, IV, V).

TABLE II

S. S. WHITE SILICATE IN PLASTIC STATE

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)								
	NO SERUM OR SALIVA ADDED			SERUM ADDED			SALIVA ADDED		
	a†	b‡	c§	a	b	c	a	b	c
Staphylococcus pyogenes var. *aureus*	0.46	0.40	0.46	0.44	0.36	0.40	0.54	0.43	0.43
Staphylococcus pyogenes var. *albus*	0.92	0.86	0.83	0.40	0.30	0.36	0.52	0.40	0.43
Streptococcus viridans	0.24	0.23	0.26	0.0	0.0	0.0	0.38	0.33	0.30
Escherichia coli	0.34	0.33	0.30	0.28	0.26	0.26	0.28	0.23	0.26
Lactobacillus acidophilus var. *odontolyticus**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus casei	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†a—Plates examined at end of 48 hours' incubation at 37.5° C.

‡b—Plates were placed in refrigerator (9° C.) for 12 to 15 hours, then incubated and examined after 48 hours.

§c—Plates incubated for 168 hours at 37.5° C. and examined at the end of this period.

TABLE III

RESIN + 2 PER CENT SnF_2 IN PLASTIC STATE

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)								
	NO SERUM OR SALIVA ADDED			SERUM ADDED			SALIVA ADDED		
	a†	b†	c†	a	b	c	a	b	c
Staphylococcus pyogenes var. *aureus*	0.0	0.0	0.0	0.0	0.0	0 0	0.0	0.0	0.0
Staphylococcus pyogenes var. *albus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Streptococcus viridans	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Escherichia coli	0.16	0.10	0.20	0.0	0.0	0.0	0.0	0.0	0.10
Lactobacillus acidophilus var. *odontolyticus**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus casei	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†a, b, and c—see legends for Table II.

TABLE IV

RESIN + 2 PER CENT NaF IN PLASTIC STATE

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)								
	NO SERUM OR SALIVA ADDED			SERUM ADDED			SALIVA ADDED		
	a†	b†	c†	a	b	c	a	b	c
Staphylococcus pyogenes var. *aureus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Staphylococcus pyogenes var. *albus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Streptococcus viridans	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Escherichia coli	0.16	0.10	0.0	0.0	0.0	0.0	0.18	0.0	0.0
Lactobacillus acidophilus var. *odontolyticus**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus casei	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†a, b, and c—see legends for Table II.

The addition of sterile horse serum or sterile human saliva produced results which varied with the material, as well as the test organism. In experiments using *Staph. pyogenes* var. *aureus* as the test organism, the addition of serum or saliva decreased the width of the zone with one of the commercial silicates

TABLE V

FLUORON IN PLASTIC STATE

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)								
	NO SERUM OR SALIVA ADDED			SERUM ADDED			SALIVA ADDED		
	a†	b†	c†	a	b	c	a	b	c
Staphylococcus pyogenes var. *aureus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Staphylococcus pyogenes var. *albus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Streptococcus viridans	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Escherichia coli	0.10	0.16	0.10	0.0	0.0	0.0	0.22	0.16	0.20
Lactobacillus acidophilus var. *odontolyticus**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus casei	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†a, b, and c—see legends for Table II.

but not with the other. In experiments with *Staph. pyogenes* var. *albus*, a zone of varying width was observed with the commercial silicates, the commercial zinc phosphate cement and an experimental zinc phosphate to which CaF_2 was added. The addition of sterile horse serum to the medium decreased the antibacterial action of the two zinc phosphate cements while a definite increase was noted in the case of one of the silicates.

TABLE VI

S. S. WHITE ZINC PHOSPHATE CEMENT

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)								
	NO SERUM OR SALIVA ADDED			SERUM ADDED			SALIVA ADDED		
	a†	b†	c†	a	b	c	a	b	c
Staphylococcus pyogenes var. *aureus*	0.0	0.0	0.0	0.0	0.0	0.0	0 0	0.0	0.0
Staphylococcus pyogenes var. *albus*	0.44	0.36	0.40	0.18	0.0	0.20	0.18	0.26	0.20
Streptococcus viridans	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Escherichia coli	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus acidophilus var. *odontolyticus**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus casei	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†a, b, and c—see legends for Table II.

In experiments using *Str. viridans* as the test organism, a double zone effect was seen, a zone of complete inhibition immediately adjacent to the pellet surrounded by a zone of decreased growth. The addition of sterile horse serum decreased the inhibitory effect of the silicates while sterile human saliva seemed to enhance their antibacterial action.

When *Esch. coli* was used as the test organism, a zone of decreased growth immediately surrounding the pellets was found with the silicates. However, resins which contained fluorides produced a minute, clear zone surrounding the pellets. The addition of sterile horse serum or sterile human saliva to the media produced variable results.

No significant changes in zones of inhibition were observed when plates were stored at 9° C. for 12 to 15 hours and then incubated at 37.5° C. for 48 or 72 hours or when they were directly incubated for 168 hours.

TABLE VII

EXPERIMENTAL ZINC PHOSPHATE CEMENT + 5 PER CENT CaF_2 IN PLASTIC STATE

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)								
	NO SERUM OR SALIVA ADDED			SERUM ADDED			SALIVA ADDED		
	a†	b†	c†	a	b	c	a	b	c
Staphylococcus pyogenes var. *aureus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Staphylococcus pyogenes var. *albus*	0.60	0.66	0.50	0.20	0.30	0.23	0.30	0.50	0.13
Streptococcus viridans	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Escherichia coli	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus acidophilus var. *odontolyticus**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lactobacillus casei	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†a, b, and c—see legends for Table II.

Powders and Liquids.—As with the hardened pellets, no zones of inhibition were observed against any of the test organisms with the 11 cement powders tested.

Representative results obtained with each type of liquid can be seen in Table VIII. Only those containing phosphoric acid inhibited the growth of *Staph. pyogenes* var. *aureus*, *Staph. pyogenes* var. *albus*, *Str. viridans* and *L. acidophilus* var. *odontolyticus*. No zones of inhibition were noticed when *L. casei*, *Esch. coli*, and *Cl. sporogenes* were used as the test organisms. Kadon* and FluorOn liquids proved to be quite inert, failing to exert antibacterial action against all the test organisms except *Esch. coli* where a small zone with reduced growth was seen.

TABLE VIII

CEMENT LIQUIDS

ORGANISM USED	MEAN ZONE OF INHIBITION (CM.)			
	S. S. WHITE SILICATE	KADON	FLUORON	S. S. WHITE ZINC PHOSPHATE CEMENT
Staphylococcus pyogenes var. *aureus*	3.3	0.0	0.0	4.5
Staphylococcus pyogenes var. *albus*	3.7	0.0	0.0	4.5
Streptococcus viridans	3.7	0.0	0.0	3.9
Escherichia coli	0.0	2.3†	2.5†	0.0
Lactobacillus acidophilus var. *odontolyticus**	2.3	0.0	0.0	5.5
Lactobacillus casei	0.0	0.0	0.0	0.0
*Clostridium sporogenes**	0.0	0.0	0.0	0.0

*In the case of *L. acidophilus* var. *odontolyticus* and *Cl. sporogenes*, plates were examined after 72 hours' incubation.

†Reduced growth rather than complete inhibition.

DISCUSSION

The results obtained with the silicates were more or less in agreement with those of McCue[9] and Shay[11] and their co-workers. McCue, using *Micrococcus aureus* and *Esch. coli* as test organisms, observed marked germicidal action

*Kadon liquid was employed with all of the experimental resin materials.

with silicate cement. Shay, however, reported that silicate cement was among the least effective inhibitory material tested against *L. casei, Bacillus bifermentans,* and *Str. viridans.* Results of the present investigation demonstrate that silicate cement exerted antibacterial action against *Staph. pyogenes* var. *aureus, Staph. pyogenes* var. *albus, Esch. coli* and *Str. viridans,* but was ineffective when *L. casei, L. acidophilus* var. *odontolyticus,* and *Cl. sporogenes* were used. The antibacterial action of zinc phosphate cement was slight.[10, 12]

Considering the entire group of materials, generally a decrease in the zone of inhibition was observed with the addition of sterile horse serum to the medium, and this also corroborated the results of McCue.[9]

No zones of inhibition were observed when the pellets were transferred to freshly inoculated plates or when they were allowed to harden for 18 to 24 hours and then embedded in the inoculated plates. Early research in this area suggested the germicidal efficiency of a cement to be dependent on the liquid or on the presence of soluble salts.[8] Since no antibacterial action was observed with the cement powders and only those mixing fluids containing phosphoric acid inhibited the growth of *Staph. pyogenes* var. *aureus, Staph. pyogenes* var. *albus, Str. viridans* and *L. acidophilus* var. *odontolyticus,* it is highly probable that phosphoric acid was the agent primarily responsible for the antibacterial action of silicate cement when studied in the plastic state. Moreover, failure of hardened pellets to inhibit bacterial growth, coupled with lack of any appreciable antibacterial action observed with the fluoride-containing materials even when still plastic, lend further support to this theory.

SUMMARY

The antibacterial effects of certain fluoride-containing dental materials against 7 different microorganisms have been investigated. The powders, liquids, plastic and hardened pellets of the materials were all studied.

These data indicate that the addition of low concentrations of fluorides to resins or zinc phosphate cement will not inhibit growth of the microorganisms here tested. Certainly, the results of this study give increased support to the theory that the lower incidence of recurrent caries associated with the silicate restoration is due to decreased solubility of the tooth structure which is in apposition to the restoration.

The materials listed in decreasing order of their antibacterial action in the plastic state were the two commercial silicates, zinc phosphate cement containing 5.0 per cent calcium fluoride, a commercial zinc phosphate cement, and resins containing fluoride. Addition of sterile horse serum or human saliva to the medium produced variable results. There was no inhibition of bacterial growth on transfer of the pellets to freshly inoculated plates. Likewise, no effect was observed when the hardened pellets or cement powders were used. Mixing fluids containing phosphoric acid were strongly antibacterial whereas the resin liquid was ineffective. The implications of these results have been discussed.

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THE ROLE OF ACIDITY OF SALIVA IN THE NUTRITION OF ORAL ACIDOGENS

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THE experiments described below indicate that bacterial proteolytic enzymes play an essential role in the production from saliva of the amino acids necessary for the growth of the common oral acidogens. The experiments demonstrate the role of salivary acidity in this production and provide an explanation of the effect of glucose on the establishing of acidogens in the mouth.

The amino acid requirements of the streptococci and lactobacilli have been discussed by several authors, including Koser and Thomas,[1] Anderson and Elliker,[2] and Niven and Sherman,[3] and the requirements of the two groups in general are similar in that both require at least 7 amino acids, and usually more, for growth and acid production. The free amino acids in saliva have been discussed by Kirch, Kesel, O'Donnell, and Wach,[4] and Woldring.[5] Weisberger[6] has shown that saliva incubated with glucose produces amino acids sufficient for the growth of *L. acidophilus*. By incubating saliva in the presence of glucose, he found an increase in amino nitrogen and amino acid CO_2 and little change in ammonia; in the absence of glucose he found an increase in ammonia.[7] Proteolytic enzymes in saliva have been reported by Willstatter[8] and Voss.[9] It was shown by Dreizen, Reed, and Spies[10] that the action of trypsin or amylopsin on salivary mucin releases amino acids sufficient for the growth of *L. acidophilus*.

METHOD

The general plan of the experiments was to add saliva, or a salivary fraction incubated for various periods of time, to a solution containing all the nutrients, except amino acids, necessary for the growth of *L. casei*, to inoculate with *L. casei*, and to measure the growth and acid production.

The solution used was the synthetic medium of Henderson and Snell[11] modified by a fivefold increase in the vitamin concentration and, in the bioassay experiments, by the omission of amino acids. In each case, stock solutions of the various nutritional components were mixed with a clear or nearly clear neutralized and centrifuged saliva or salivary fraction. The mixture was adjusted to pH 6.8 and to a volume of 5.0 ml., autoclaved 7 minutes at 15 pounds pressure, inoculated, and incubated at 37° C. for 72 hours.

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Inoculation was made with a 0.1 ml. suspension of *L. casei* which had been grown for about 24 hours in 5.0 ml. of tomato juice-yeast broth, washed 3 times with sterile saline, and resuspended in 5.0 ml. of sterile saline. Seventy-two hour growth was measured in the Evelyn colorimeter using a 660 filter and acid production was measured by titration with 0.1 normal sodium hydroxide using bromthymol blue indicator. The Beckman potentiometer was used for pH determinations. In most cases, the tests were done in duplicate and a control with the full complement of amino acids as casein hydrolysate and tryptophane was run simultaneously. Maximum acid production of the complete medium was usually 8.0 ± 0.4 ml. of 0.1 normal acid. The amount of saliva or salivary fraction in all cases corresponded to 1.0 ml. of saliva.

The paraffin-stimulated saliva, or salivary fractions, prepared in various ways, were incubated at 37° C. for periods from 0 to 188 hours with or without toluene. Submaxillary and sublingual salivas were collected with the Schneyer[12] appliance, and parotid saliva with the Curby[13] cup. Saliva was reconstituted from the fractions according to figures reported by Schneyer and Levin[14] in terms of the relative rates of flow of the 3 main salivary fractions: submaxillary 69 per cent, sublingual 5 per cent and parotid 26 per cent. Tests for sterility were made on blood agar plates and in thioglycollate broth after 48 hours of aerobic incubation at 37° C.; the submaxillary and sublingual were found to be slightly contaminated. This contamination is important inasmuch as the experiments in general showed that bacterial enzymes were responsible for the liberation of the greater part of the amino acids necessary to promote growth of the *L. casei* in the bio-assay. The numbers of organisms found in the contaminated specimens were, however, so small that any contribution by them to the proteolytic enzyme systems under consideration was deemed negligible.

Saliva was collected from one subject before and after a period of 16 hours during which time 45,000 units of penicillin as troches were taken by mouth along with periodic streptomycin rinses containing one gram of streptomycin each. During this time the total bacterial count fell from about 270,000 to 1,000 colonies per milliliter when cultured aerobically on blood agar plates. No inhibitory effects of residual antibiotics were observed in the assay for amino acids.

RESULTS

The results are summarized in Table I. As shown by Weisberger,[6] it can be seen that the full complement of amino acids and/or peptides is not present before incubation. The very small growth at 0 incubation time indicates, within the limits of error of the method, the absence of a complete set of amino acids. This complement of amino acids appears when the saliva is incubated in the presence of toluene, or in the absence of toluene, provided the saliva is acid. In general, the production of amino acids continues at a slow rate during the course of even the most prolonged incubation period. It can also be seen that, in general, the enzymatic activity is predominantly in the centrifuged fraction and that there is considerable substrate in the supernatant.

TABLE I

ACID PRODUCED BY *L. casei* WHEN MEDIUM WAS SUPPLIED WITH INCUBATED SALIVARY FRACTIONS AS THE ONLY SOURCE OF AMINO ACIDS

SUBJECT	SALIVA	HOURS OF INCUBATION OF SALIVA	ACID PRODUCED BY *L. casei* (ml. OF 0.1 N)
1	Whole	23 + T*	1.9
	Whole	23 − T*	0.1
2	Whole	22 + T	2.2
		43 + T	2.7
		0 − T	0.3
3	Whole	188 + T	3.4
4	Whole	186 + T	2.8
5	Whole	43 + T	4.6
		0 − T	0.3
6	Whole	168 + T	2.5
	Heated supernatant + unheated centrifugate	168 + T	1.8
	Heated centrifugate + unheated supernatant	168 + T	1.5
2	Whole	168 + T	3.4
	Heated supernatant + unheated centrifugate	168 + T	2.9
	Heated centrifugate + unheated supernatant	168 + T	2.2
5	Whole	168 + T	5.6
	Heated supernatant + unheated centrifugate	168 + T	5.3
	Heated centrifugate + unheated supernatant	168 + T	3.3
5	Whole	118 + T	5.3
	Whole before antibiotics	45 + T	5.5†
	Whole after antibiotics	64 + T	1.4‡
	Heated supernatant + unheated centrifugate after antibiotics	64 + T	1.0
	Heated centrifugate + unheated supernatant after antibiotics	64 + T	0.9
5	Whole	168 + T	3.7
	Sublingual + submaxillary§	168 + T	0.5
	Sublingual + parotid§	168 + T	0.6
	Submaxillary + parotid§	168 + T	0.6
	Submaxillary, parotid sublingual§	168 + T	0.6
6	Whole	20 + T	1.6
	Whole heated	20 + T	0.2
	Submaxillary, parotid, sublingual	20 + T	0.2
	Heated submaxillary, parotid, sublingual	20 + T	0.2
	Pooled		
	Whole 7.5‖ 7.7¶	23 + T	2.5
	Whole heated	0 − T	0.3
	Whole + acetate buffer 4.4 4.4	0 − T	0.6
	Whole + acetate buffer 4.4 4.4	4 − T	1.3
	Whole + acetate buffer	23 − T	2.1
	Whole + glucose (5%) 7.7 7.7	0 − T	0.4
	Whole + glucose (5%) 7.7 4.8	4 − T	0.7
	Whole + glucose (5%) 7.7 4.0	23 − T	1.4

*+ T = Toluene; − T = No toluene.
†Ca 270,000 colonies per ml.
‡1,000 colonies per ml.
§Slightly contaminated.
‖Initial pH.
¶pH after incubation.

That most of the proteolytic activity is attributable to bacterial enzymes is shown by the experiments on reconstituted saliva in which the bacterial count was greatly reduced, and by the experiments on saliva after treatment of the subject with antibiotics. In both cases, a large reduction in total bacteria was accompanied by a large reduction in proteolytic activity.

DISCUSSION

That the amount of proteolytic enzymes present in human saliva is a factor limiting the rate of acid production in the presence of glucose is probably the explanation of the work of Fosdick and Rapp[15] in which the addition of papain or trypsin increased the speed with which saliva-glucose mixtures decalcified enamel.

It has been shown by Shklair, Englander, Stein, and Kesel[16] that the filling or extraction of all carious teeth reduced the lactobacillus count, and by Bradel and Blayney[17] that the lactobacillus count dropped considerably in edentulous mouths. These findings are consistent with the inference from our experiments that only as saliva is retained in inaccessible places in the mouth do amino acids accumulate in it sufficient for the nutrition of lactobacilli and streptococci.

The fact that in the absence of inhibitors there is a production of amino acids sufficient for the growth of lactobacilli and streptococci, only when the saliva is acid, suggests that the production of acid from ingested carbohydrate greatly enhances the production of amino acids in places where the dilution of locally retained saliva by freshly secreted saliva is low, and could very well explain the relationship existing between dietary carbohydrate intake and the level of oral lactobacilli.[18-20] It also seems possible that the localization and extension of carious lesions are not to be explained solely by the inaccessibility of certain areas to the buffering action of saliva, but that there is also involved the accumulation of amino acids in such areas and their subsequent diffusion to adjacent areas.

SUMMARY

1. Saliva incubated in the presence of toluene or acid furnishes a mixture of amino acids and/or peptides sufficient to fulfill the amino acid requirements of *L. casei*.

2. Reconstituted saliva and saliva collected after treatment with antibiotics show a considerable drop in amino acid production coincident with the almost complete absence of bacteria.

3. The relevance of these findings to the localization and propagation of the carious lesion and to the ecology of acidogenic organisms in the mouth is discussed.

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BACTERIOLOGIC STATUS OF PERIAPICAL TISSUE IN 150 CASES OF INFECTED PULPLESS TEETH

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THE bacteriologic status of the periapical tissue has been studied by cultures in one of several ways, namely, (1) by way of the root canal, (2) by extracting the tooth and culturing the root surface or attached periapical soft tissue, (3) by means of a trocar and cannula, and (4) by making a window in the labial plate of bone, as in root resection. We shall consider each of these methods in turn.

1. *Root Canal.*—Culturing of the periapical tissue by way of the root canal is not a reliable method. The root canal itself may not be sterile even though the periapical tissue is sterile. The method developed by Hedman[1] in which a sterile cannula is introduced into the root canal, and a sterile wire is then carried through the cannula into the periapical tissue for taking the culture, circumvents this objection.

2. *Extraction.*—The taking of cultures from extracted teeth or from the tooth socket has been the most common method for determining the bacteriologic status of the periapical area. It is extremely difficult, if not impossible at times, to prevent contamination of the root of the tooth during extraction. Fish[2] has aptly described the difficulty in these words: "A pool of blood wells up round the edge of the gum before the tooth gives way, and this blood becomes contaminated from the crowns of the surrounding teeth and finally is smeared over the root as it leaves the socket." In addition, Tunnicliff and Hammond[3] and others have pointed out that, in the course of extracting a tooth, positive and negative pressure is created which alternately forces and sucks the microorganisms present in the gingival area up along the root surface. Appleton[4] has said: "It is doubtful if a tooth can be extracted by ordinary methods without contaminating its apex with saliva; and it is by no means certain that contamination can be avoided even when the operator is meticulously trying to avoid contamination. Consequently, in the long run it might be a good thing if we wiped the slate clean and started from scratch."

3. *Trocar and Cannula.*—The trocar and cannula when introduced through the labial alveolar plate of bone simply provides a sterile avenue for obtaining a sample for culturing. This method was originally introduced by Coriell,[5] and refinements in the instrument and technic were made by Grossman.[6] Of 32 cases examined by the author in this manner, 27 gave negative cultures from

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both trocar and cannula. In one case, cultures from both trocar and cannula were positive, in 2 cases positive cultures were obtained from the trocar, and negative from the cannula, while in 2 other cases the reverse was found.

The trocar and cannula method (apicostomy) has this limitation: one cannot always be sure of having reached the periapical tissue unless the operator feels the instrument drop into the rarefied area of destroyed bone.

4. *Resection.*—The method which is most reliable for examining periapical tissue to determine whether it is sterile or not is that of root resection. It was first suggested by Garvin[7] and used for this purpose shortly afterward by Berwick.[8] Burket[9] also used this method for examining necropsy material. It has the advantage over other methods in the certainty that material for culturing will be taken from the periapical area and there is less likelihood for contamination than by any other method. Also, one can select specific areas for culturing, for example, the root apex, bone, soft tissue, etc. The disadvantage is that an operation with its potential postoperative sequelae must be carried out in order to obtain samples for study.

The material studied in this report was gathered over a period of time while routinely doing root resections as demonstrations before undergraduate students. In all cases, significant areas of rarefaction were present associated with pulpless teeth.

METHOD

The material for culturing was obtained from pulpless teeth with areas of rarefaction varying from about 5 to 20 mm. in diameter. All teeth had been pulp-tested prior to resection and did not react to the electric current. The roentgenographic diagnosis was either subacute or chronic abscess, granuloma, or cyst. In most cases, the root resection operation was carried out by the immediate method, for example, no prior treatment had been given the root canal, and both treatment and filling of the root canal were done immediately preceding the operation. Under the circumstances, there was an element of chance for contaminating the periapical tissue during instrumentation of the root canal. In other cases, the root canal was filled after two negative cultures had been obtained, and the root resection operation was carried out the next day, or at most, within a few days. In all cases, the area to be operated upon was blocked off with sterile gauze and the teeth, mucosa, gingivae, and lips were swabbed with untinted tincture of Metaphen. A control culture was then taken of the mucosa in the operative field. Another control culture, prior to entering the periapical area, was taken of the labial plate of bone as soon as the mucoperiosteal flap was raised. A window was then prepared in the bone by removing a section of the labial plate overlying the root tip. A culture was taken of the exposed root tip or of the adherent soft tissue. Following curettement, another culture was taken of the osseous bed. In all, 4 cultures were taken. This procedure was followed in 86 cases. In 64 additional cases, the same procedure was followed except that separate cultures were taken of the exposed root tip, and of the soft periapical tissue adjacent to it, such as, granulomatous or cystic tissue, making a total of 5 cultures.

All cultures were incubated in brain heart infusion broth (Difco) aerobically at 37° C., and examined after 48 hours and again after one week, at which times records were made of the presence or absence of growth. The incubated culture tubes were examined by a trained bacteriologic technician. No study was made of the types of organisms, when recovered, as we were interested in knowing only whether organisms were present or not.

EVALUATION OF DATA

The data in 41 of the 150 cases had to be discarded for the following reasons: the mucosa (control) was positive in 22 cases; the labial plate of bone (control) was positive in 10 cases; there was an incomplete number of cultures taken in 9 cases. In 10 cases, cultures of the labial plate of bone were positive but in 6 of these cases all other cultures, both of the mucosa and of the periapical tissue, were negative. These data are not included in the evaluation. This left a total of 109 cases in which valid data were available. Of this number, all cultures, both controls and those taken of the periapical area, were negative in 93 cases (85.3 per cent). A breakdown of the data is given in Table I.

TABLE I
CULTURES TAKEN PROGRESSIVELY DURING ROOT RESECTION

	1 MUCOSA		2 LABIAL PLATE		3 ROOT TIP		4 SOFT TISSUE		5 OSSEOUS BED		ALL CULTURES	IN-COM-
	POS.	NEG.	POS.	NEG.	POS.	NEG.	POS.	NEG.	POS.	NEG.	NEGATIVE	PLETE
Series 1	14	67	1	80	9	72			1	80	57	5
Series 2	8	52	9	51	4	56	6	54	6	54	36	4
Total	22*	119	10*	131	13	128	6	54	7	134	93	9*

*Indicates contaminated controls or incomplete cases (total of 41) which were not included in the evaluation.

DISCUSSION

A question might very well be asked regarding the bacteriologic status of the root canal in these cases. It was originally assumed that the root canal was infected in all cases because an area of rarefaction was present. In most of the cases, an immediate root resection was done without taking a culture of the root canal. In other cases, a two-stage operation of root canal treatment, followed later by root resection, was done. In the latter group, control cultures taken of the root canal in 49 cases prior to treatment yielded 9 negative cultures (18.2 per cent) and 40 positive cultures (81.7 per cent). By far the larger number of cases showed infection of the root canal. The question might therefore be asked why an area of rarefaction was present or persisted in those cases where negative cultures had been obtained from the root canals. It is entirely likely that in such cases where trauma had caused death of the pulp, the breakdown of tissue produced irritation of the periapical bone resulting in its eventual destruction in that area. In other cases, a bacterial irritant may have been initially present in the root canal causing destruction of periapical bone from irritation of the toxic products. An accumulation of toxic products in the

root canal, however, may result in destruction of the microorganisms themselves. The periapical exudate may then diffuse into the open root canal, become stagnant and break down, forming products which continue to irritate the periapical tissue and so interfere with repair even though the bacterial irritant is no longer present.

Fish is of the opinion, based upon histologic study, that periapical bone (like bone elsewhere in the body) is sterile because microorganisms cannot survive for any appreciable length of time in the presence of polymorphonuclear cells which are called into action as soon as microorganisms make their appearance. The question now arises, in such cases where microorganisms were recovered from the periapical tissue: Were they residents of this area or were they introduced into this area during mechanical instrumentation of the root canal or from trauma of burring the bone and root tip during the resection operation itself? It is also possible that in some of the cases where a chronic abscess was present, necrotic bone formed a protective area for the microorganisms so that they were out of reach of the polymorphonuclear cells. Clinically, necrotic bone, as manifested by a roughened, pumice-like surface, is seldom seen during a resection operation.

SUMMARY

1. Of 150 cases in which cultures were taken serially of the periapical tissue during root resection, reliable data were available in 109 cases.

2. Of the 109 cases, all cultures taken of the periapical tissue, as well as the controls, were negative in 93 cases (85.3 per cent).

3. Speculation is made regarding the presence and the significance of microorganisms in the periapical tissue in such cases where they were recovered.

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DIFFERENCES IN THE MORPHOLOGY AND SIZE OF THE TEETH OF A CARIES-SUSCEPTIBLE AND A CARIES-RESISTANT STRAIN OF RATS

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THE basic morphologic pattern of teeth in human beings and in animals is apparently determined by a genetic mechanism (Dahlberg,[1] and Hunt, Hoppert and Rosen[2]). Paynter and Grainger[3] have recently established that in the rat the genetic potential for molar tooth morphology can be significantly altered by varying an animal's diet during development of the teeth; i.e., an altered developmental environment can modify the predetermined genetic pattern of the teeth.

Caries susceptibility in human molar teeth (as is the case with rats) is very much greater for the fissured surfaces than for smooth surfaces. Since susceptibility to pit and fissures caries seems to be largely confined to a definite proportion of the population (Reid and Grainger[4]), it might be presumed that susceptible and immune people differ from one another in terms of the shape of the fissures in their teeth. This difference, if present, could result from inheritance, from differences in tooth environment during development, or from a combination of both.

Thus, it seemed of interest to explore the possibility that the greater predisposition to dental caries in a group of susceptible rats might, to a considerable extent, be associated with morphologic differences between their teeth and those of resistant animals. Some indication that this may be the case has been shown by Kifer, Hunt, Hoppert, and Witkop,[5] who demonstrated a wider opening into the fissures in the teeth of the Hunt-Hoppert susceptible rats than in the Hunt-Hoppert resistant rats.

The present study was, therefore, set up to try to measure differences (presumably inherited) in morphology and size of the molar teeth in two groups of rats, one caries-susceptible and the other caries-resistant, which had been selectively bred on the basis of caries susceptibility in the laboratories in the Harvard School of Dental Medicine.

This study has been supported in part by funds from the Associate Committee on Dental Research of the National Research Council of Canada, and from the Canadian Dental Association.

The development and maintenance of the caries-resistant and caries-susceptible strains of rats, of which the subjects in this study were representative, have been to a large extent supported at the Harvard School of Dental Medicine by contract Nonr 1866(01) with the office of Naval Research.

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METHOD

Young rats, 12 to 14 days old, which had been raised from either the caries-resistant or caries-susceptible strain of rats at the Harvard School of Dental Medicine, were decapitated, and the heads fixed in 10 per cent formalin. Each head was identified as male or female by removing one ear from an animal of either sex, then identified numerically by a tag bearing its parents' Harvard laboratory number, and designated as Group I or Group II depending on the caries susceptibility of the strain to which the animal belonged. The heads were then shipped to the laboratory of the Division of Dental Research, University of Toronto, for processing. The sex and caries susceptibility were

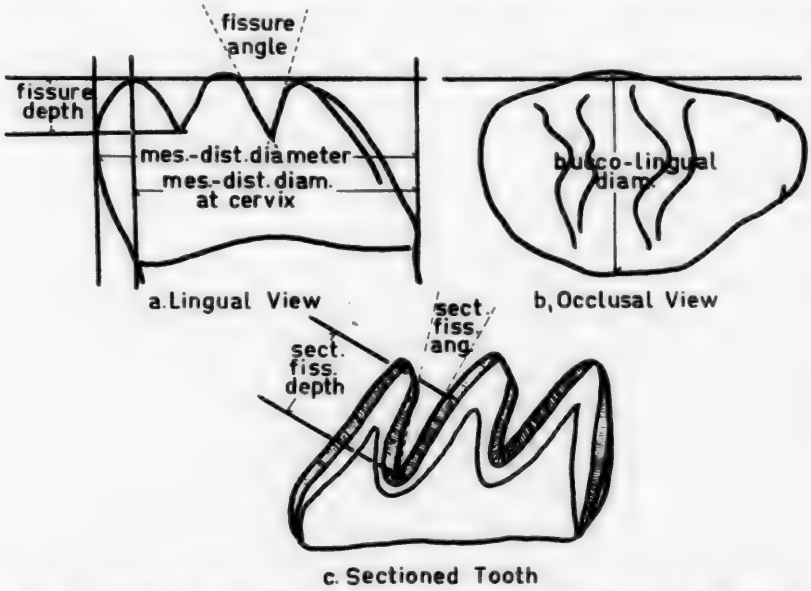

Fig. 1.—Outline drawings of the lingual and occlusal aspects of the upper right first molar of a rat and of the cut surface of a hemisectioned tooth showing the method of measuring the tooth characteristics.

unknown in Toronto until the morphologic examination and statistical analysis were completed. The distribution of the animals by number and sex, and according to their litter and group, is shown in Table I. The total numbers shown in this table were not used for all analyses since a few of the specimens were lost during processing.

Littermates of some of the animals listed in Table I were maintained on a cariogenic diet at the Harvard laboratories for a minimum of 92 days to verify their caries susceptibility (see Table VII). Only those litters were sampled for caries susceptibility which contained more offspring than were required for ideal sampling of the morphologic characteristics.

For purposes of studying the teeth, the upper right and upper left first molars were dissected from each head. The upper right molars were cemented to a stiff wire so that photographs could be made of the occlusal and lingual

surfaces of each tooth after the manner previously described (Paynter and Grainger[3]). Standard enlargements were made from the negatives on a non-shrink photographic paper.* The magnification factor was 30 times the original tooth diameter. Measurements made from these pictures included the greatest mesiodistal diameter of the crown, the mesiodistal diameter at the cervix, the buccolingual diameter, and the visible depth and angle of the sides of the mesio-occlusal fissure as seen from the lingual aspect of the whole crown (Fig. 1, *a* and *b*).

The left maxillary first molar was also cemented to a stiff wire, this time so that the lingual surface was positioned at right angles to the long axis of the wire. Then, using a diamond abrasive disc, the lingual surface was ground on a plane approximately parallel to that bisecting the crown both mesio-distally and cervico-occlusally. Grinding was continued until approximately half the tooth was removed, leaving the buccal half-crown still cemented to the wire holder. The cut surface of the tooth was then photographed, and the negative enlarged, under the same conditions as those described above (Fig. 2).

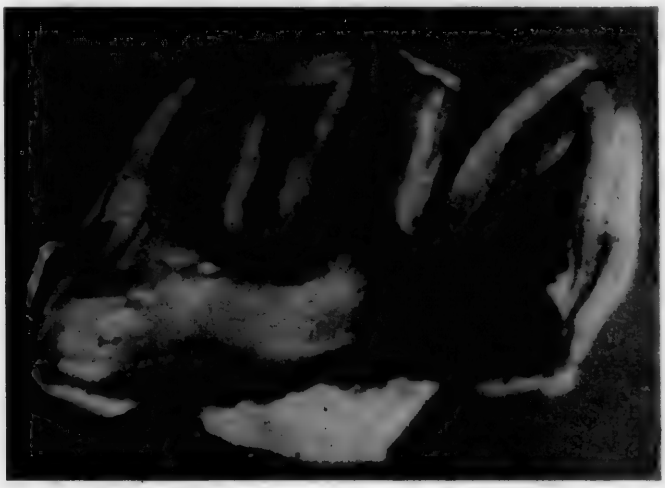

Fig. 2.—A photograph of the cut surface of a rat upper left first molar which has been ground approximately halfway through the crown.

These photographs were used to measure both the depth of the mesio-occlusal fissure (measured from a line from the center of the occlusal portion of the mesial cusp and at a right angle to the distal side of the fissure, to the base of the fissure [see Fig. 1, *c*]), and also the angle of the sides in the lower third of the same fissure.

The plane to which the crowns were cut could not be perfectly standardized, but any variation in line of cut was assumed to be random for purposes of analysis, since the identity of the specimens was unknown to the assistant who prepared, mounted, and photographed the specimens, and who made the measurements used for analysis.

*Kodak Resisto-Rapid enlarging paper.

TABLE I
DISTRIBUTION OF TWO GROUPS OF 12 LITTERS OF RATS ACCORDING TO THE NUMBER AND SEX OF THE ANIMALS IN EACH LITTER

GROUP I (CARIES-RESISTANT)				GROUP II (CARIES-SUSCEPTIBLE)			
LITTER NO.	NO. OF ANIMALS			LITTER NO.	NO. OF ANIMALS		
	SEX A	SEX B	TOTAL		SEX A	SEX B	TOTAL
4	3	5	8	1	3	3	6
6	3	4	7	2	3	4	7
7	4	4	8	3	3	4	7
8	3	4	7	5	3	2	5
9	5	2	7	14	3	3	6
10	3	4	7	15	3	5	8
11	1	4	5	16	3	4	7
12	5	3	8	18	2	4	6
13	4	3	7	21	3	1	4
17	3	5	8	22	2	1	3
19	2	5	7	23	1	3	4
20	4	4	8	24	5	2	7
Total	40	47	87	Total	34	36	70

FINDINGS

The litter means for each measurement have been arrayed in Table II according to groups. The results of the statistical analyses of the measurements are shown in Tables III to VIII.

TABLE II
LITTER MEANS OF MEASUREMENTS DERIVED FROM PHOTOGRAPHS* OF THE UPPER FIRST MOLAR TOOTH OF A CARIES-RESISTANT AND A CARIES-SUSCEPTIBLE GROUP OF RATS

LITTER NO.	NO. ANIMALS	MESIO-DISTAL DIAMETER OF CROWN	MESIO-DISTAL DIAMETER OF CROWN AT CERVIX	BUCCO-LINGUAL DIAMETER OF CROWN	DEPTH OF FISSURE, LINGUAL VIEW	ANGLE OF FISSURE, LINGUAL VIEW	DEPTH OF SECTIONED FISSURE	ANGLE OF SECTIONED FISSURE
Group I. Caries-Resistant								
4	8	106.31	96.19	58.62	17.50	50.31	21.14	12.69
6	7	99.42	91.68	53.86	24.25	38.08	22.50	11.21
7	8	96.00	87.94	54.87	19.94	43.75	22.21	10.00
8	7	99.83	92.83	55.71	18.00	53.25	21.50	11.75
9	7	101.86	93.86	57.86	22.07	42.07	26.71	14.29
10	7	104.00	96.79	57.93	20.21	51.14	24.71	9.00
11	5	98.80	92.50	55.40	20.00	51.30	23.70	11.50
12	8	99.44	91.63	56.25	17.81	46.50	23.42	8.33
13	7	101.64	92.14	56.79	23.57	40.79	--	--
17	8	105.69	95.25	56.50	20.56	42.81	25.12	9.94
19	7	101.64	92.79	54.79	21.43	41.86	22.57	14.86
20	8	100.94	92.38	55.44	19.00	52.81	21.94	11.19
Group II. Caries-Susceptible								
1	6	99.50	87.50	57.38	12.38	48.50	18.00	--
2	7	94.83	84.75	55.00	16.58	41.00	20.63	7.13
3	7	98.50	87.14	53.21	14.14	50.86	19.50	4.80
5	5	97.90	89.50	55.70	12.70	48.40	19.20	8.90
14	6	101.17	92.17	55.92	13.67	46.67	20.00	8.17
15	8	97.31	88.81	55.69	13.94	45.38	21.00	4.00
16	7	101.07	88.43	55.86	16.71	45.21	20.57	3.50
18	6	102.83	91.33	57.08	14.75	48.50	22.75	3.33
21	4	97.25	89.00	54.88	14.63	47.75	19.63	7.25
22	3	90.17	83.33	50.66	13.33	52.00	20.13	3.75
23	4	96.13	86.63	56.50	15.00	40.63	21.83	2.67
24	7	100.71	91.43	56.56	14.71	48.14	20.43	5.79

*Magnification factor—×30.

To test for differences in tooth measurements between the sexes, an analysis of variance was carried out for each value. No differences in these measurements of statistical significance were found between sexes and, hence, for the examination of differences between groups, the sex identity of the animals was ignored.

In Table III are presented the results of the analysis of variance which was carried out for each measurement, in order to test the homogeneity of the litters within the two groups. In the caries-resistant group, significant differences were found between litters for all measurements except the angle of the sectioned fissure (differences in buccolingual diameter were of borderline significance). On the other hand, within the susceptible group, significant differences were found for only 4 of the 7 measurements (as opposed to 6 out of 7 for the resistant group). There was no significant difference between susceptible litters in the measurements of the angle of the fissure sides, whether measured from the lingual view or from the section, or of the depth of the sectioned fissure.

TABLE III
ANALYSIS OF VARIANCE FOR SEVEN MEASUREMENTS FROM THE UPPER FIRST MOLARS OF A CARIES-SUSCEPTIBLE AND A CARIES-RESISTANT GROUP OF RATS

UPPER FIRST MOLAR MEASUREMENT	SOURCE OF VARIATION	GROUP				HETEROGENEITY OF GROUP MEAN SQUARES AT 2 PER CENT LEVEL
		RESISTANT		SUSCEPTIBLE		
		D.F.	MEAN SQUARE	D.F.	MEAN SQUARE	
Mesiodistal diameter of crown	Within litters	74	12.56	56	11.27	N.S.
	Between litters	11	66.14	11	51.86	N.S.
			F 5.27**		F 4.60**	
Mesiodistal diameter of crown at cervix	Within litters	74	13.94	56	10.07	N.S.
	Between litters	11	42.53	11	35.43	N.S.
			F 3.05**		F 3.52**	
Buccoling. Diameter of crown	Within litters	75	7.97	56	3.34	Sign.
	Between litters	11	15.11	11	14.05	N.S.
			F 1.90†		F 4.21**	
Depth of fissure, lingual view	Within litters	74	10.16	56	4.45	Sign.
	Between litters	11	32.63	11	9.82	N.S.
			F 3.21**		F 2.21*	
Angle of fissure, lingual view	Within litters	73	50.73	55	71.30	N.S.
	Between litters	11	188.70	11	58.41	N.S.
			F 3.72**		F 0.82	
Depth of sectioned fissure	Within litters	65	6.61	48	5.93	N.S.
	Between litters	10	20.14	11	6.15	N.S.
			F 3.05**		F 1.04	
Angle of sectioned fissure	Within litters	61	29.80	49	19.65	N.S.
	Between litters	10	28.35	11	23.95	N.S.
			F 0.95		F 1.22	

* and **—Litter differences in same group significant at 5 per cent level and 1 per cent level, respectively.
†Borderline significance.

For all measurements, except the angle of the fissure from the lingual view, the variation between and within litters was less for the susceptible group than it was for the resistant group. This difference in variation between the

groups was significant statistically in the case of the buccolingual diameter and the depth of the fissure from the lingual view (Table III).

The results of the analysis for differences in tooth measurements between the two groups is presented in Table IV and illustrated in Fig. 3. It may be observed that, on the average, the teeth of the susceptible rats were smaller than those of the resistant animals in mesiodistal crown diameter and mesiodistal cervical diameter. The cervical measurement difference was the more significant statistically. The average difference in buccolingual diameters, while following the same trend (the susceptible measurement was smaller) was not

TABLE IV
ANALYSIS OF DIFFERENCES IN UPPER FIRST MOLAR MEASUREMENTS BETWEEN A CARIES-SUSCEPTIBLE AND A CARIES-RESISTANT GROUP OF RATS

UPPER FIRST MOLAR MEASURMENT	COMPUTATION	GROUP		"t" TEST BETWEEN GROUPS	5 PER CENT CONFIDENCE LIMITS ON DIFFERENCE
		RESISTANT	SUSCEPTIBLE		
Mesiodistal diameter of crown	Group average	101.42	98.58		
	No. of litters	12	12	t = 2.28*	
	Variance of litters as units†	9.54	9.02	d.f. 11	+0.14 to +5.58
	S.E. of average	0.89	0.87		
Mesiodistal diameter of crown at cervix	Group average	93.00	88.80		
	No. of litters	12	12	t = 4.16**	
	Variance of litters as units	6.13	6.16	d.f. 11	+1.98 to +6.42
	S.E. of average	0.71	0.72		
Buccoling. diameter of crown	Group average	56.20	55.50		
	No. of litters	12	12	t = 1.10	
	Variance of litters as units	2.12	2.71	d.f. 11	−0.70 to +2.10
	S.E. of average	0.42	0.48		
Depth of fissure, lingual view	Group average	20.17	14.51		
	No. of litters	12	12	t = 8.07**	
	Variance of litters as units	4.70	1.71	d.f. 11	+4.12 to +7.20
	S.E. of average	0.63	0.38		
Angle of fissure, lingual view	Group average	46.18	46.79		
	No. of litters	12	12	t = 0.33	
	Variance of litters as units	27.21	10.16	d.f. 11	−4.72 to +3.50
	S.E. of average	1.63	0.92		
Depth of sectioned fissure	Group average	23.25	20.50		
	No. of litters	11	12	t = 4.49**	
	Variance of litters as units	2.61	1.65	d.f. 10	+1.39 to +4.11
	S.E. of average	0.49	0.37		
Angle of sectioned fissure	Group average	11.35	5.25		
	No. of litters	11	12	t = 6.24**	
	Variance of litters as units	4.58	6.44	d.f. 10	+3.83 to +8.27
	S.E. of average	0.65	0.73		

* and **—Difference between group means significant at 5 per cent and 1 per cent level, respectively.

†Variance of litters as units is based on weighted sum of squares because of differences in litter size.

statistically significant. The average depth of the mesio-occlusal fissure was less in the teeth of the susceptible rats, and this difference was significant whether measured from the lingual view of the whole crown, or from the sectioned tooth. There was no significant difference between the two groups in the average angle of the sides of the mesio-occlusal fissure when measured from the exterior lingual view, but the angle of the sides of the fissure in the sectioned teeth was, on the average, smaller in the susceptible rats; i.e., the sides of the fissure in its basal third were more nearly parallel (Fig. 3).

Summarizing, the teeth from the caries-susceptible rats, on the average, appeared to be smaller in mesiodistal diameter (particularly at the cervix), had shallower mesio-occlusal fissures, and the sides of these fissures were more nearly parallel at the base (Table IV).

Fig. 3.—A drawing of the cut surface of hemisectioned teeth from a caries-resistant and a caries-susceptible strain of rats, superimposed on squared paper to show the typical differences in size and shape of the teeth between the two groups.

Since the mesiodistal diameter, the fissure depth, and the angle of the sides of the fissure in the sectioned teeth differed significantly between the groups, it was important to know if these factors were correlated or whether they were independently related to caries susceptibility. Because the mesiodistal diameter of the crown was shown graphically to be closely related to the mesiodistal diameter at the cervix, and since the cervical dimension difference between groups was more highly significant (Table IV), the latter dimension was considered to be more fundamental and was used in computing the correlations. The first degree correlations and the partial correlations are presented in Table V. The only correlations that approached significant magnitude in both groups of animals were those between the mesiodistal diameter of the crown at the cervix, and the depth of the sectioned fissure (Table V, r_{13} and $r_{13.2}$).

Since the correlations between the angle and the depth of the fissure appeared generally low, these factors were believed to be separately associated with caries susceptibility in the animals.

TABLE V
LINEAR CORRELATIONS BETWEEN MEASUREMENTS OF THE UPPER FIRST MOLAR TEETH OF RATS FROM A CARIES-SUSCEPTIBLE AND A CARIES-RESISTANT STRAIN

FACTORS COMPARED (SOURCE)	GROUP: RESISTANT — WITHIN LITTERS D.F. 60 (.25 = 5% SIG.)	RESISTANT — BETWEEN LITTERS D.F. 9 (.60 = 5% SIG.)	SUSCEPTIBLE — WITHIN LITTERS D.F. 46 (.29 = 5% SIG.)	SUSCEPTIBLE — BETWEEN LITTERS D.F. 10 (.58 = 5% SIG.)
r_{12}	−.08	−.04	−.06	+.38
r_{13}	+.24†	+.32	+.36*	+.22
r_{23}	+.02	−.01	−.02	−.45
r_{14}	+.26*	−.20	+.13	−.26
r_{24}	−.06	+.36	+.12	−.34
$r_{12.3}$	−.09	−.04	−.06	+.55
$r_{13.2}$	+.24†	+.32	+.39*	+.47
$r_{23.1}$	+.04	<+.01	<+.01	−.37

r = the product moment coefficient of correlation between factors indicated as below:
1. Mesiodistal diameter of the crown at cervix.
2. Angle of sectioned fissure.
3. Depth of sectioned fissure.
4. Depth of fissure, lingual view.

*Significant at 5 per cent level.
†Of borderline significance.

Next, in order to test whether the depth of the fissure was a simple derivation of tooth diameter with which it appeared correlated, the mutually adjusted differences in mesiodistal diameter at the cervix and in fissure depth between the two groups of rats were tested by an analysis of covariance. The results are given in Table VI. When the fissure depths were adjusted in terms of regression on tooth size, the differences in fissure depth between the two groups remained significant, although significance was reduced from the 1 per cent to the 5 per cent level of confidence. On the other hand, when the cervical crown diameters were adjusted in terms of regression on fissure depth, the difference

TABLE VI
ANALYSIS OF VARIANCE AND ANALYSIS OF COVARIANCE OF AVERAGE MESIODISTAL DIAMETERS AT THE CERVIX, AND THE AVERAGE FISSURE DEPTH (IN SECTION) OF THE UPPER FIRST MOLAR TEETH BETWEEN THE CARIES-SUSCEPTIBLE AND THE CARIES-RESISTANT STRAIN OF RATS

CHARACTERISTIC	SOURCE	ANALYSIS OF VARIANCE — TEST OF MEANS IN TABLE IV — D.F.	ANALYSIS OF VARIANCE — TEST OF MEANS IN TABLE IV — MEAN SQUARE	ANALYSIS OF COVARIANCE — TEST OF MEANS ADJUSTED BY LINEAR REGRESSION — D.F.	ANALYSIS OF COVARIANCE — TEST OF MEANS ADJUSTED BY LINEAR REGRESSION — MEAN SQUARE FOR ERRORS OF ESTIMATE
Depth of sectioned fissure	Groups	1	200.0	1	72.6
	Litters	21	12.0	20	11.5
			F = 21.7**		F = 6.3*
Mesiodistal diameter of crown at cervix	Groups	1	622.8	1	134.7
	Litters	21	34.6	20	33.2
			F = 18.0**		F = 4.1 nonsig.

* and **—Significant at 5 per cent and 1 per cent level, respectively.

between the groups was reduced to nonsignificance. On the basis of those results, it was tentatively concluded that while the depth of the fissure as measured in this study could be a function of tooth size, the fissure depth was the factor most strongly associated with the caries susceptibility of the tooth—the mesiodistal diameter of the crown at the cervix being perhaps only secondarily associated with caries susceptibility.

Having reduced the independent anatomic factors which appeared to be strongly associated with susceptibility or resistance to dental caries, to a measure of the depth and the angulation of the sides in the lower one third of the mesio-occlusal fissure (both measurements from the sectioned tooth), an attempt was made to clarify the relative importance of these two factors and to determine the nature of their relation to caries. The data available for this purpose was limited since this aspect of the investigation had not been anticipated at the time the study was designed. Caries scores for the purpose outlined were derived from observations on the teeth of littermates which had been kept on a caries-inducing diet for a period of either 160 or 92 days, depending on whether the animals belonged to the caries-resistant or susceptible group. The caries data are presented in Table VII, along with the mean fissure depths and angle as measured from the sectioned molars.

TABLE VII

MEAN DEPTH AND MEAN ANGLE OF SECTIONED FISSURES OF THE UPPER FIRST MOLAR TEETH OF RATS COMPARED TO CARIES SCORES OF LITTERMATES KEPT ON A CARIES-INDUCING DIET FOR 160 AND 92 DAYS, RESPECTIVELY, FOR RESISTANT AND SUSCEPTIBLE STRAINS

LITTER NO.	NO. OF ANIMALS MEASURED	MEAN DEPTH OF SECTIONED FISSURE	MEAN ANGLE OF SECTIONED FISSURE	NO. OF LITTERMATE ANIMALS FROM WHICH CARIES WAS RATED	NO. OF CARIOUS MOLARS	NO. OF CARIOUS LESIONS	EXTENT OF CARIOUS LESIONS
Resistant Group							
4	8	21.4	12.7	2	5.5	5.5	8.5
6	6	22.1	13.1	3	1.6	1.6	3.3
7	7	22.2	10.0	2	0	0	0
8	2	22.2	11.8	1	0	0	0
9	7	26.7	14.3	1	0	0	0
10	7	24.7	9.0	1	0	0	0
12	6	23.4	8.3	2	1.0	1.0	1.0
17	8	25.1	9.9	2	1.0	1.0	1.0
19	7	22.6	14.9	2	0	0	0
20	8	21.9	11.2	4	2.2	2.2	2.5
Group Averages		23.3	11.5		1.4	1.4	2.0
Susceptible Group							
1	1	18.0	0	2	7.0	11.5	28.5
2	4	20.6	7.1	2	5.5	9.5	16.0
3	5	19.5	4.8	2	7.0	11.2	28.0
15	8	21.0	4.0	2	8.0	16.0	55.0
16	7	20.6	3.5	1	10.0	14.0	34.0
Group Averages		20.4	4.4		7.2	12.3	32.1

Although the caries data were recognized to have strict limitations, the regressions of caries scores on fissure depth and on fissure angle were calculated, and are shown graphically in Fig. 4. Because of the very wide differences between the means of the susceptible and resistant groups about which the

litter scores are assembled, however, there may be a graphic illusion as to the strength of the relationship between dental caries on the angle and depth of the fissure. In Fig. 4, the dotted lines indicate the least squares fitted regressions of the caries scores on the other factor specified, for each of the two groups of animals. The slopes of these lines are in no sense the same for the two groups, and hence the group means cannot be thought to lie along a linear regression line common to both groups.

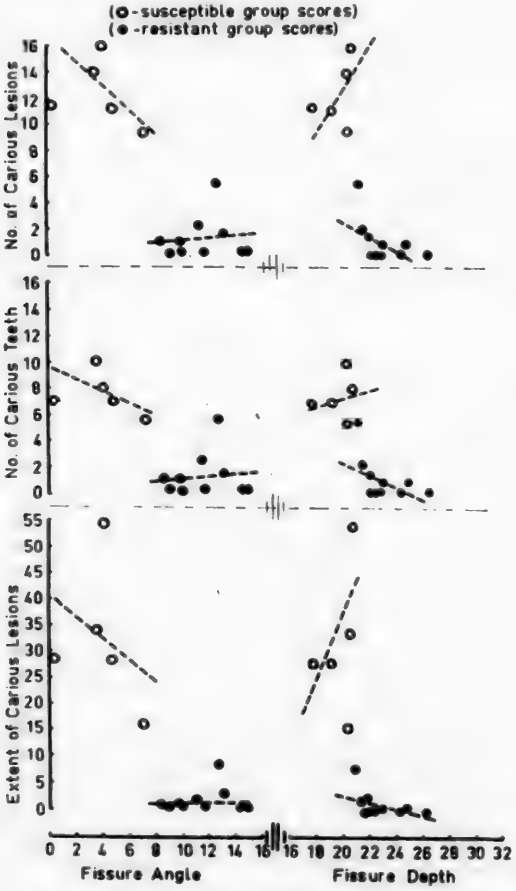
(O-susceptible group scores)
(●-resistant group scores)
No. of Carious Lesions
No. of Carious Teeth
Extent of Carious Lesions
0 2 4 6 8 10 12 14 16
Fissure Angle
16 18 20 22 24 26 28 30 32
Fissure Depth

Fig. 4.—A graph to show the nature of regressions on caries scores (number of carious teeth, number of carious lesions and extent of carious lesions) on the depth and angle of the mesio-occlusal fissure of a caries-resistant and a caries-susceptible group of rats.

DISCUSSION

It is difficult to discuss the results of this study, relating to tooth morphology and dental caries, without inferring causation. It is not intended, however, that this concept should arise from the discussion. A morphologic characteristic cannot "cause" a disease process. For example, a fissure on the surface of a tooth cannot cause decay in that tooth, although the presence of the fissure,

at least in human beings, is a very great predisposing factor to the decay. This thought should be borne in mind when reading the following discussion which relates primarily to correlations between certain anatomic characteristics of rat molars, and caries resistance or susceptibility in the animals.

Neither the caries-resistant nor the caries-susceptible groups of rats under study were pure strains with respect to caries susceptibility or to morphologic characteritics of their teeth. Real differences in caries susceptibility were observed within both groups under comparison. The data for the two current groups, along with data from many other laboratory evaluations of caries-proneness in these genetically-derived strains, suggests that their proneness to caries has not been developed to the absolute degree to be desired. The caries tendency is modified by the relative strength of various characteristics which are probably transmitted through the genes. Likewise, in the comparison of the morphologic characteristics, within the litters in each group there was a rather wide variation in the measurements between animals (for example, the angle of the fissure, Table III). Except for the angle of the fissure from the lingual view, the observed variation within litters of the susceptible group was less than in the resistant group. This observation suggests that the process of selective breeding for caries had produced a purer caries-susceptible strain in terms of tooth morphology. The greater uniformity of the caries-susceptible strain is also noted in connection with the incidence and extent of carious lesions, where the variability evidenced in this and other studies is always less for the susceptible than for the resistant strain.

The report of Kifer, Hunt, Hoppert, and Witkop,[5] that the fissures in the lower molar teeth of caries-susceptible rats of the Hunt-Hoppert strain were wider than those in the teeth of resistant rats of the same strain, appears to be at variance with the results shown in the present study. These workers made no measurement of upper teeth of their animals. Their plane of tooth section was buccal to the midline (see Fig. 1, Kifer and co-workers[5]) and fissure width was defined as the distance from the posterior lip of a fissure along a perpendicular to the line representing the anterior fissure wall. In the present report, no comparable measurement was made, and measurements were made only on upper first molar teeth.

Since the present data indicate that, on the average, the angle of the sides of the mesio-occlusal fissure in the sectioned teeth from the susceptible rats was narrower than the angle in teeth from the resistant animals (Table IV), it might be assumed that this indicates a narrower fissure throughout its total depth. This assumption is not necessarily correct, since the measurement defined in this study as the angle of the sectioned fissure was made only in the basal third of the fissure. This was the angular measurement which differed significantly between groups (Table IV). The angular measure of the same fissure taken from the lateral view of the whole tooth, which is probably an index of the upper third of the fissure, did not differ between groups. In fact, the group average for the susceptible animals was even slightly larger than that for those resistant. These results indicate the possibility that the outline of the whole fissure at the midline of the tooth may also differ between groups;

the fissure shape in the susceptible animals being more like a funnel, with a narrow base and wide-open mouth, and that in the resistant rats more smoothly V-shaped. Thus, a measurement in this study similar to the one made by Kifer and associates[5] could have agreed with their findings.

Correlations between the measurements of the depth of the fissure and the angle of its sides were generally low (Table V) and, thus, each of these individual characteristics of the same fissure were thought to act as a separate factor in connection with caries susceptibility.

The present analysis is not believed to be sufficient grounds for entirely ruling out the possibility that the mesiodistal size of the crown rather than the depth of the fissure is the more important in relation to caries susceptibility (Table VI). Because of the fact that tooth size can be rather easily modified by dietary intake during the developmental period of the tooth (Paynter and Grainger[3]), the size of the tooth may possibly be a measure of its "quality." There is a real need for further qualitative analyses of tooth structure in this sort of investigation, especially where the studies are carried to the logical conclusion of a determination of the influence of altered tooth size on caries susceptibility. We recognize that some reservation of this idea is forced because of the lack of significance in the difference in buccolingual diameter between the two groups in this study. If the crown diameter does reflect tooth "quality," why should one dimension of the crown reflect this property and not the other? However, the point is open to clarification in future investigations, and lack of significance in buccolingual diameter in the present instance may prove to be simply a sampling vagary.

The data on caries scores which have been used in this study are more limited in value than they might have been had this aspect of the investigation been anticipated when the study was originally planned. The resistant littermates, from which caries data were obtained, were retained on the caries-inducing diet for a longer period than were the susceptible animals. Therefore, the former ratings were indeterminably higher than they should be for direct comparison between the two groups. Normally, however, different caries evaluation times are unavoidable in two strains with such marked differences in proneness to caries. If the resistant rats had been sacrificed with the susceptible rats at 92 days, on the basis of past experience it is known that none of the resistant rats would have had clinically discernible carious lesions. Hence, no measure of the variations in susceptibility within the resistant strain would be available. On the other hand, if the susceptible rats had been sacrificed at 160 days with the resistant rats, the caries incidence in all the susceptible rats would have approached and, in many of the subjects, reached a maximum several weeks prior to sacrifice. In this case again, there would have been no expression of the relative susceptibility of the litters within the susceptible population. Even more important than the different times of sacrifice of the two populations, the average caries ratings obtained from the few available littermates are no doubt only a rough assessment of the true caries susceptibility of the animals from which measurements of tooth morphology were obtained (dependent on the strength of the correlation in caries scores within litters).

However, with the available data, when the regressions of caries scores on both fissure depth and fissure angle were computed (see Fig. 4), the slopes for the lines for the two animal groups were not the same and, thus, it was concluded that the group means could not lie along a linear regression line common to both the susceptible and resistant groups. Out of this observation two opposing possibilities suggested themselves: first, that the depth of the fissure and the angle of its sides were not factors primarily asociated with the observed difference in caries susceptibility between the two groups of rats, but that these anatomic features were correlated to the unknown vital factor and at the same time had a modifying influence on caries scores, and the extent or nature of the influence differed for each group. The second alternative was that the regression of caries scores on these two factors was not linear, and that the linear regressions for the separate groups, illustrated in Fig. 4, really represented slopes at different points on an unknown curve.

No decision regarding these alternatives could be derived from the scanty data available, but there was some further exploration of the two lines of thought. The relationship of the caries scores to the depth and to the angle of the fissure (standardized with respect to each other) within the separate groups was expressed in terms of the standard partial correlation coefficients* as shown in Table VIII. The very high values for the susceptible group are relatively unimportant since they are associated with only two degrees of freedom. It was helpful, however, to confirm that the relative magnitude and sign (+ or −) of the standardized regressions were similar to those of the unstandardized regressions illustrated in Fig. 4.

Under the first of the two suggested hypotheses, i.e., that the fissure depth and angle have only a secondary modifying influence on dental caries susceptibility, their possible influence is suggested from Table VIII. For the susceptible animals, caries may increase directly as the fissure depth and inversely as the fissure angle. For the resistant group, caries may become manifest where the fissure depth (and tooth size) is unusually small, but is little influenced by further increase in the fissure angle.

On the other hand, if, as is suggested in the second hypothesis, there does exist a primary curvilinear relationship between caries activity and the factors being considered, then the slopes of the partial regression coefficients in Table VIII are illustrative of the two dimensional curve in the high and the low regions. The nature of the multiple relationship from which caries activity might be predicted has been suggested in the freehand sketch in Fig. 5. Chance variation in caries scores for litters should be envisaged as variation above or below the contour surface. The observed values for the litter means from the present study have been added merely to illustrate the meaning of the relationship. Computing the exact contour or testing its goodness of fit would require a very large amount of new data.

*The standard partial regression coefficients were computed from the weighted sums of squares by the product moment correlation method. Weights were used equal to the harmonic mean of the number of animals used for measurement means, and the number used for caries scores given in Table VII.

It would seem of paramount importance in the future to study the tooth characteristics of parent animals to try to determine whether the marked variation within litters is due to the complexity of the inherited factors dictating tooth morphology, or more simply due to having inadvertently mated animals which possessed the tooth characteristics to a varying degree. Using the fissure angle as the example, it was apparent from the raw data that although, on the average, the susceptible animals had smaller fissure angles, it was still possible to select individual animals with fissure angles a high as 19 degrees,

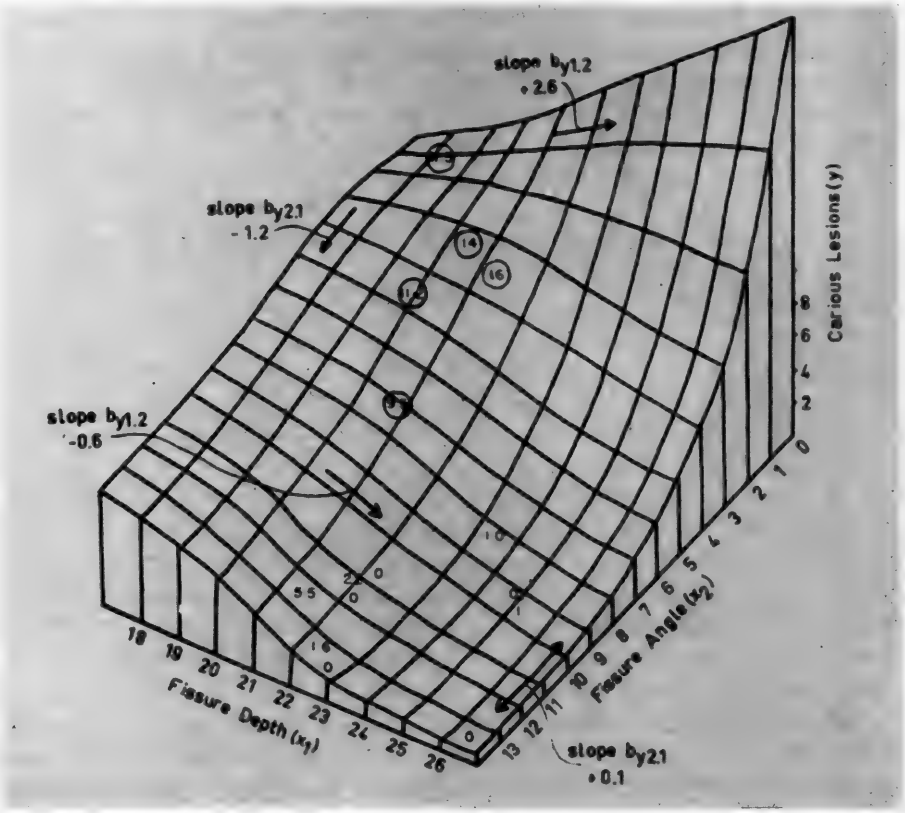

Fig. 5.—A freehand sketch to illustrate a multiple curvilinear relationship between fissure depth and angle, and dental caries susceptibility in rats as suggested by the partial regression coefficients of Table VIII. Circled numbers on the contoured surface indicate observed caries scores from susceptible animals; plain numbers—the scores from resistant rats.

which is wider than the average for the resistant group. If a single tooth were extracted for fissure morphology classification prior to assignment of animals for mating, it would be possible to select parent animals with fissure angles close to zero. Thus, breeding for caries susceptibility might be made much more efficient, and the litters produced under such a system might constitute a purer strain which would reproduce itself indefinitely. Similar steps could be taken

to purify strains regarding fissure depth or tooth size. However, if as is suggested above, the genetic mechanism is more complex than this would imply, or if the tooth characteristics are not the primary factors associated with caries susceptibility, then further refinement of strains might well prove impossible.

This suggested proposal of extracting a single tooth for morphologic examination prior to assigning animals within groups in an investigation if applied to caries studies, should vastly improve the possibilty of clarifying the relation of caries susceptibility to tooth morphology. In spite of the wide variation observed between littermates for tooth characteristics, significant differences were detected between the caries-resistant and caries-susceptible groups. This points to a very strong relationship between dental caries and tooth morphology and size. The nature of these relationships, as suggested in Fig. 4, are expected to be greatly clarified when tooth measurements and caries scores are available from the same animals. Such data are now being collected.

TABLE VIII

STANDARD PARTIAL CORRELATION COEFFICIENTS AND PARTIAL REGRESSION COEFFICIENTS EXPRESSING THE REGRESSION OF CARIES SCORES ON FISSURE DEPTH AND ON FISSURE ANGLE, RESPECTIVELY, FOR THE MESIO-OCCLUSAL FISSURE OF THE UPPER FIRST MOLAR OF CARIES-SUSCEPTIBLE AND CARIES-RESISTANT RAT STRAINS

		CARIES SCORE		
DEFINITION OF COEFFICIENT	RAT GROUP	AVERAGE CARIOUS TEETH PER LITTER	AVERAGE CARIOUS LESIONS PER LITER	AVERAGE EXTENT OF CARIES PER LITTER
Standard partial correlation of caries and fissure depth	Susceptible (d.f. 2)	+0.81	+0.79	+0.98*
	Resistant (d.f. 7)	−0.49	−0.55	−0.13
Standard partial correlation of caries and fissure angle	Susceptible (d.f. 2)	−0.80	−0.78	−0.94
	Resistant (d.f. 7)	+0.04	+0.16	+0.17
Partial regression for caries on fissure depth	Susceptible	+1.2	+2.6	+14.5
	Resistant	−0.6	−0.6	−0.2
Partial regression for caries on fissure angle	Susceptible	−0.6	−1.2	−6.6
	Resistant	+0.03	+0.1	+0.2

*Correlation significant at 5 per cent level.

The independence (particularly within litters—Table II, r_{23} and r_{24}) of tooth measurements which differed significantly between groups suggested the possibility of devising a scheme for developing a differential diagnosis for caries susceptibility. Applying this idea to human beings, it has been demonstrated through epidemiological studies in Ontario Health Units (Jarrett, Grainger, Paynter, and Nikiforuk[6]) that a much closer prediction of caries susceptibility is possible by combining available information on such factors as fluoridation history, lactobacillus count, and tooth size, than is possible by any single prediction. Animal studies, of course, have the great advantage that the oral environmental attack produced by the diet can be controlled, thus permitting accurate examination and possible separation of genetic and developmental environmental effects on the teeth.

SUMMARY

Measurements of the size and certain morphologic characteristics of the upper first molar teeth have been compared between a caries-susceptible and a caries-resistant group of rats (Harvard strains). Measurements included the greatest mesiodistal diameter of the crown, the mesiodistal diameter of the crown at the cervix, the greatest buccolingual diameter of the crown, and the depth and the angle of the sides of the mesio-occlusal fissure measured both from a lingual view of the whole crown and from a hemisection of the tooth.

No significant differences were found for any of the tooth characteristics measured, between male and female animals.

Within the caries-resistant group significant differences were found between litters for all measurements except the angle of the sectioned fissure. Within the caries-susceptible group no differences of significance were found between litters for the fissure angle, whether measured from the lingual view of the crown or from the section, or for the fissure depth. The other measurements did differ significantly between litters in this group. Except for the angle of the fissure from the lingual view, the variation between litters for each characteristic was less in the susceptible group than in the resistant group.

When the differences in measurements were tested between susceptibility groups, all were found to be significant except the buccolingual diameter of the crown and the angle of the fissure sides as measured from the lingual view. The difference in mesiodistal diameter at the cervix was of more significance than was the difference in greatest mesiodistal crown diameter.

The angle and the depth of the mesio-occlusal fissure were concluded to be separate factors apparently related to caries susceptibility. While fissure depth seemed to be related to the size of the crown at the cervix, it was also established that of these two factors, fissure depth seemed more strongly related to caries susceptibility.

Suggestions have been made as to the nature of possible relationships between the depth and angle of the sides of the fissure, and caries susceptibility.

The capable assistance of Mr. G. Levenstadt in preparing the specimens and undertaking the measurements is hereby gratefully acknowledged.

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SALIVARY GLAND FUNCTION IN THE RAT

III. Protease and Arginase Activity of Submaxillary Glands and Whole Saliva

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EVIDENCE has been presented in previous studies suggesting that the activity of different endocrine organs, principally the thyroid gland, is responsible for alterations in dental caries incidence in the rat.[1-3] Further investigations have shown that this endocrine control of caries activity is undoubtedly mediated through the salivary glands, although the exact mechanism is not known.[4-8] Histologic changes have been observed in the submaxillary glands which suggest that salivary gland function might be responsible for the differences in caries incidence. A proteolytic enzyme (protease) has been identified in rat submaxillary gland extracts and has been found to be related to thyroid gland activity.[6] Another proteolytic enzyme, arginase, has been studied by Kochakian, Endahl, and Hall[9] who reported that its activity in mouse salivary glands was decreased by many of the cortical steroids, while thyroxine had no effect on arginase activity of the salivary glands.

The presence of both protease and arginase in salivary gland tissue and the fact that their activity may be altered by endocrine dysfunctions have suggested that further investigation of these enzymes might provide fundamental information on the endocrine-dental caries relationship. Since the presence of a specific enzyme in gland extracts does not prove that the enzyme is secreted in the saliva, it was important to investigate the enzymatic nature of whole saliva for the presence or absence of protease and arginase and to determine whether or not their presence is altered by endocrine dysfunction.

EXPERIMENTAL

The present study was divided into several parts, each designed to help clarify this complex problem of salivary gland-enzyme activity under the influence of endocrine dysfunction. All of the animals in this investigation were albino rats of the Sprague-Dawley strain maintained on raised wire-bottom cages and fed a stock corn diet* and distilled water ad libitum. The numbers of the animals in each portion of the study are shown in the table summarizing that portion of the experiment.

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*The diet used in this study consisted of: yellow corn grits, 52.7 per cent; ground yellow whole corn, 11.3 per cent; powdered whole milk, 30.0 per cent; alfalfa, 4.8 per cent; iodized sodium chloride, 1.0 per cent; and irradiated yeast, 0.2 per cent.

Part I.—This portion of the study was divided into 3 groups: (1) a control, (2) animals receiving 10 μg daily of sodium thyroxine injected subcutaneously, and (3) animals receiving 0.1 per cent propylthiouracil in the diet. Ten weeks after the beginning of the drug administration, the animals were anesthetized with Nembutal (35 mg./Kg. body weight) and injected subcutaneously with 10 mg. of pilocarpine nitrate per kilogram of body weight in order to collect saliva for determination of protease and arginase activity. Protease activity was determined by methods previously described.[10] The determination of arginase activity of the salivary gland tissue itself and in saliva is a modification of the method of Friedman and Becker.[11] In preparation for the determination of arginase activity, the glands from one side of an animal were immediately weighed and placed in cold (7° C.) redistilled water (0.04 ml./mg. of tissue) overnight. The following day, tissue homogenates were prepared for analysis with a Potter-Elvehjem homogenizer. An arginine substrate was prepared freshly by dissolving 9 Gm. of L-arginine monohydrochloride in 3.2 ml. of 9 N carbon dioxide-free sodium hydroxide, and made to a volume of 50 ml. with distilled water. This arginine solution had a concentration of 0.85 M and a pH of 9.4. A total of 1.0 ml. of the arginine substrate was mixed with 1 ml. aliquots of the homogenized salivary gland extract or, in the case of the analysis of whole saliva, with 1 ml. aliquots of pilocarpine-stimulated saliva, and incubated at 25° C. for exactly 10 minutes. Digestion was stopped by adding 2 ml. of 15 per cent trichloroacetic acid to each tube. The tubes were shaken and allowed to stand at room temperature for 15 minutes. A second sample was prepared by mixing 1 ml. of tissue homogenate, 2 ml. of trichloroacetic acid, and 1 ml. of arginine substrate in order to determine pre-formed urea in the tissue. A blank consisting of 1 ml. of distilled water, 2 ml. of trichloroacetic acid, and 1 ml. arginine substrate was also prepared. All 3 tubes were centrifuged at 2,000 rpm for 20 min., and to 2 ml. of the supernatant from each tube was added 15 ml. of a sulfuric-phosphoric acid mixture (90 ml. of concentrated H_2SO_4 and 270 ml. of 85 per cent H_3PO_4 diluted to 1 L. with distilled water). Color was developed by adding 0.5 ml. of α-isonitrosopropiophenone (3.0 Gm. per 100 ml. of 95 per cent ethyl alcohol) to 10 ml. of each of the mixtures. The tubes were then placed in the dark and boiled for 1 hour. The amount of color formed was determined at once in a Klett photoelectric colorimeter using filter No. 54. Arginase activity was expressed as colorimetric units developed by the urea by subtracting the pre-formed urea from the total urea liberated after correcting for the blank.

Part II.—Adult male rats (5 months of age) were divided into 3 groups, the first serving as controls while each animal of the second group was injected with 750 μc of carrier-free radioactive iodine (I^{131}). The third group received daily subcutaneous injections of 10 μg of thyroxine. At the termination of the study period of 30 days, the animals were anesthetized and saliva collected for protease and arginase determinations as previously described.

Part III.—Male rats (60 days of age) were divided into 3 groups, the first group serving as controls while each animal of the second group was given 500 μc of carrier-free radioactive iodine (I^{131}). Seven days later, the animals in this latter group were given 10 μg of thyroxine daily for 4 additional weeks. The third group of animals also received 500 μc of I^{131} and, in addition, 0.25 per cent potassium iodide in the drinking water. After the experimental period of 4 weeks, the animals were sacrificed; the right submaxillary glands were removed for determination of protease activity and the opposite gland fixed in Zenker-formol for histologic examination.

Part IV.—This portion of the study was divided into 3 series. In Series I, male rats (4 months of age) were divided into 3 groups. The animals in the first group were controls. The animals in the second group were hypophysectomized at the age of 4 months, while animals in the third group were hypophysectomized but, in addition, received 25 mg. of testosterone twice weekly and 10 μg of thyroxine daily throughout the experimental period of one month. At the termination of the experiment the animals were sacrificed, the combined submaxillary-sublingual glands were removed and arginase activity determined. The opposite glands were prepared for histologic examination.

Series II consisted of male rats, 3½ months of age, divided into 3 groups. The first group consisted of controls, the second of animals receiving 10 μg of thyroxine daily, while the third group received 0.1 per cent propylthiouracil in the diet. At the termination of the experimental period of 28 days, the animals were sacrificed and the glands removed for arginase activity and histologic examination.

Series III consisted of animals which were divided into 3 groups. The first group served as controls while the second group received between 5 and 10 mg. of desiccated thyroid in the diet daily depending upon the animal's body weight and drug tolerance. The third group received 0.1 per cent propylthiouracil in the diet. At the end of the experimental period of one month, the animals were sacrificed and the salivary glands removed for arginase activity and for histologic examination.

Part V.—One group of adult male rats with a mean body weight of 351 Gm. were injected subcutaneously with 0.6 ml. per kilogram of body weight of a 10 per cent aqueous solution of pilocarpine nitrate. A second untreated group served as controls. The pilocarpine animals were sacrificed 1, 2, and 4 hours after administration of the sialogogue. In a second portion of Part V, rats of the same sex and strain, having a mean body weight of 372 Gm. were injected subcutaneously with 25 mg. per kilogram of body weight of a 2.5 per cent aqueous solution of pilocarpine nitrate. An additional untreated group served as controls. In this portion of the study, the animals were sacrificed at periods of 1, 2, 4, 6, and 8 hours following the administration of the drug. Control animals were sacrificed only at the beginning of the study.

When the animals were sacrificed, the right combined submaxillary and sublingual glands were removed for protease activity, while the opposite glands were fixed in Zenker-formol solution for histologic study.

RESULTS

Part I.—Pilocarpine administered to animals receiving sodium thyroxine resulted in an increased volume of saliva when compared to controls not receiving thyroxine. Similar animals receiving propylthiouracil produced significantly less saliva ($p = 0.02$) than either the controls or the animals receiving thyroxine (Table I). The protease activity of the saliva did not differ significantly among the 3 groups. This is in contrast to studies previously reported in which it was shown that thyroxine administration significantly increases the protease activity of submaxillary gland tissue extracts.[6] In this study, however, adult animals were used, as compared to previous studies in which weanling animals were used. Propylthiouracil has been shown previously to reduce protease activity of submaxillary extracts.[6] Salivary arginase activity also was not significantly different among any of the different groups of animals.

TABLE I

THE EFFECT OF THYROXINE AND THIOURACIL ON SALIVARY FLOW AND SALIVARY PROTEASE AND ARGINASE ACTIVITY

GROUP	NO. OF ANIMALS	AVG. WT. OF ANIMALS (GM.)	SALIVA SECRETED (ML./HR.)	PROTEASE ACTIVITY/ML. OF SALIVA	ARGINASE ACTIVITY/ML. OF SALIVA
Control	7	360	2.9 ± 0.6*	156 ± 18	85 ± 31
Thyroxine	7	356	4.3 ± 1.2	160 ± 11	80 ± 24
Thiouracil	6	273	1.6 ± 0.4	185 ± 13	96 ± 28

*Standard deviation.

Part II.—The salivary flow was only slightly reduced in animals receiving I^{131} while the administration of thyroxine to radiothyroidectomized rats restored salivary volume to the level of the controls (Table II). However, these animals were also mature at the time of the thyroidectomy and, as such, cannot be directly compared with previous work. Viscosity showed an insignificant increase in the radiothyroidectomized group, and the mean viscosities for the control and I^{131}-thyroxine groups were identical.

TABLE II

THE EFFECT OF RADIOTHYROIDECTOMY AND THYROXINE REPLACEMENT THERAPY ON SALIVARY FLOW, VISCOSITY, AND PROTEASE AND ARGINASE ACTIVITY IN MATURE RATS

GROUP	NO. OF ANIMALS	WT. OF ANIMALS (GM.)	SALIVARY FLOW (ML./HR.)	AVERAGE VISCOSITY (SEC.)	PROTEASE ACTIVITY/ML. OF SALIVA	ARGINASE ACTIVITY/ML. OF SALIVA
Controls	8	343	4.6 ± 0.8	50 ± 11	101 ± 8.5	81 ± 10
I^{131}	6	348	3.6 ± 1.1	69 ± 17	140 ± 31	79 ± 27
I^{131} + thyroxine	7	323	4.7 ± 1.1	50 ± 6	214 ± 80	65 ± 20

Salivary protease activity varied widely in the different groups but did show an increase in the I^{131}-thyroxine group as compared to the control. The salivary arginase activity did not differ significantly between the three groups of animals.

Part III.—The protease activity of submaxillary gland tissue extracts was significantly greater in animals which had been radiothyroidectomized and given

thyroxine than in controls (Table III). Even though the weight of the submaxillary gland is greater in the I^{131}-thyroxine group than in the control group, the protease activity per gram of tissue is still significantly greater.

TABLE III

THE EFFECT OF THYROXINE AND POTASSIUM IODIDE IN RADIOTHYROIDECTOMIZED RATS ON PROTEASE ACTIVITY OF SUBMAXILLARY GLANDS

GROUP	NO. OF ANIMALS	AVERAGE WEIGHT OF ANIMALS (GM.)	AVERAGE WEIGHT OF GLAND (MG.)	AVERAGE PROTEASE ACTIVITY OF GLAND	
				RELATIVE	ACTIVITY/GM.
Control	5	207	186.7	3.6	4.0 ± 0.6
I^{131} + thyroxine	5	210	225.9	8.5	7.7 ± 0.9
I^{131} + potassium iodide	5	190	184.3	2.7	3.0 ± 0.6

The administration of I^{131} and potassium iodide resulted in no significant differences in protease activity of gland extracts as compared to controls. There was difference between the I^{131} animals receiving thyroxine and those receiving potassium iodide. This confirms previously reported studies and it should be noted also that the animals in this portion of the study were young animals. Furthermore, it should be noted that no differences were found in protease of whole saliva between similar groups in Part I of this study.

Part IV.—In Series I of this experiment, a slight difference in the arginase activity of submaxillary gland extracts from hypophysectomized rats was found while a decrease in activity in hypophysectomized animals receiving testosterone and thyroxine was noted (Table IV). The difference in gland activity between hypophysectomized animals receiving no supplement and those receiving the thyroxine and testosterone is significant.

TABLE IV

THE EFFECT OF THYROID FUNCTION ON ARGINASE ACTIVITY OF RAT SUBMAXILLARY GLANDS

GROUP	NO. OF ANIMALS	AVERAGE WEIGHT OF ANIMALS (GM.)	AVERAGE WEIGHT GLAND (MG.)	UNITS ARGINASE (PER WT. GLAND)
Series I				
Control	7	363	288.0	89 ± 24
Hypophysectomized	5	241	120.0	139 ± 35
Hypo. + testosterone + thyroxine	6	253	200.3	55 ± 9
Series II				
Control	5	351	297.1	80 ± 28
Thyroxine	5	337	317.0	46 ± 35
Thiouracil	5	305	237.3	89 ± 9
Series III				
Control	3	310	298.3	47 ± 27
Desiccated thyroid	2	260	342.3	18 ± 7
Thiouracil	3	256	215.2	70 ± 18

In Series II, no difference between the mean values for arginase activity in controls and animals receiving thyroxine was found (Table IV). Animals receiving propylthiouracil had essentially the same submaxillary gland arginase activity as control animals.

In Series III, the arginase activity in animals receiving desiccated thyroid resulted in a significant decrease in arginase activity while the propylthiouracil caused an increase in arginase activity as compared to controls (Table IV).

Part V.—In Series I, the relative protease activity was found to diminish over the 1-, 2-, and 4-hour periods concomitant with a diminution in the average weight of the combined submaxillary-sublingual gland (Table V). The protease activity per gram of salivary gland tissue showed no differences, however, through the 4-hour period.

TABLE V

THE EFFECT OF PILOCARPINE ON PROTEASE ACTIVITY AND GRANULAR TUBULES OF RAT SUBMAXILLARY GLANDS

TIME INTERVAL (HOURS)	NO. OF ANIMALS	AVERAGE WEIGHT OF GLAND (MG.)	AVERAGE PROTEASE ACTIVITY		AVERAGE DIAMETER OF TUBULES (μ)
			RELATIVE	ACTIVITY/GM.	
Series I					
0	4	280.6	18.0 ± 0.9	13.3 ± 1.1	44.1 ± 1.0
1	4	279.7	17.2 ± 1.2	12.6 ± 0.5	47.6 ± 2.1
2	4	261.2	15.5 ± 1.3	12.2 ± 0.9	45.8 ± 1.4
4	3	227.8	13.7 ± 1.4	12.3 ± 0.3	45.5 ± 1.7
Series II					
0	6	369.7	18.6 ± 3.5	10.4 ± 0.6	45.2 ± 0.8
1	5	375.0	17.2 ± 1.7	9.4 ± 0.5	46.5 ± 1.5
2	5	356.9	18.0 ± 1.7	10.3 ± 0.9	46.1 ± 0.9
4	5	307.8	13.6 ± 3.1	9.1 ± 0.5	45.3 ± 1.4
6	5	294.9	13.5 ± 1.5	9.4 ± 0.5	46.4 ± 1.0
8	5	319.9	15.0 ± 2.3	9.6 ± 1.1	45.6 ± 2.3

The weight of the combined submaxillary-sublingual salivary gland in Series II of this portion of the study remained essentially the same until the 2-hour interval when a precipitous decline began which was not reversed until the 8-hour period. These findings are similar to those in Series I, although no reversal of gland weight decline was found in the first series since the study continued for only 4 hours.

The relative protease activity of the glands decreased with the decreased weight of the gland at the time intervals studied. However, the activity per gram of tissue did not differ significantly.

A study was also made of the granular tubules in the submaxillary glands since it has been suggested that these are the site of the protease. These have been found to vary in diameter and granule content concomitant with certain endocrine dysfunctions which alter salivary gland-protease activity and these data have been previously reported.[6] In both Series I and Series II of this investigation, no significant changes were found in the diameter of these granular tubules at any time interval.

DISCUSSION

These investigations have been designed expressly to study protease and arginase activity in both submaxillary gland extracts and whole saliva in the rat. While the proteolytic enzyme activity of submaxillary gland extracts can be demonstrated to vary with certain endocrine dysfunctions, it has been found that there is no consistent alteration in this activity in whole saliva in animals receiving thyroxine, propylthiouracil, or in radiothyroidectomized adult rats under the conditions of the present studies.

Arginase activity of submaxillary gland extracts appears to vary inversely with proteolytic activity. It has been previously reported that hypophysectomy results in a decreased proteolytic activity of the submaxillary gland. The present studies reveal that arginase activity increases following hypophysectomy. In addition, there is some decrease in arginase activity following administration of thyroxine or desiccated thyroid and an increase following propylthiouracil administration. These findings would confirm the idea that the granular tubules of the submaxillary gland are the site of the protease, whereas the acinar cells are the source of the arginase. This may be explained assuming that, as the granular tubules increase in size when thyroxin is administered, concomitant increases in protease activity occur, suggesting that the amount of acinar tissue of gland is actually reduced, thus resulting in a decreased arginase activity. Conversely, as the granular tubules are diminished in size under the influence of hypophysectomy or propylthiouracil administration, a corresponding decrease in protease activity occurs and there is an increase in the actual amount of acinar tissue present per volume of gland, thus showing a corresponding increase in arginase activity.

The study of protease activity under the influence of pilocarpine stimulation can contribute significant information to the general problem of salivary gland function. While pilocarpine was found to increase saliva volume within a short time after administration, no obvious gross differences were seen in the appearance of the glands at any time. The gradual decline in gland weight apparently relates to an exhaustion of the secretory materials within the gland, and it is of interest that the reversal of the progressive weight loss of the gland is not noted until the 8-hour period following pilocarpine administration.

The fact that there is a significant progressive decrease in relative protease activity but no changes in protease activity per gram of gland tissue indicates that protease activity is relatively constant under stimulation of secretory activity. One would predict from these data that the protease activity of the whole saliva would not be progressively altered.

The average diameter of the granular tubules, which are thought to be the site of the proteolytic substance, does not change remarkably following pilocarpine administration. This is in contradistinction to the changes occurring following alterations in the endocrine balance where, for example, there is a very great decrease in tubular size following radiothyroidectomy, the administration of propylthiouracil or hypophysectomy.[4, 10, 12] This finding

of the constancy of the size of the tubules concomitant with lack of change of proteolytic activity following pilocarpine treatment supports the suggestion that the proteolytic substance is localized in these tubules.

SUMMARY

A series of studies has been carried out in order to investigate protease and arginase activity of the submaxillary gland and whole saliva. The data indicate that:

1. The administration of thyroxine and of propylthiouracil or thyroidectomizing doses of I^{131} does not affect the protease or arginase activity of pilocarpine-stimulated saliva.

2. The administration of thyroxine or of desiccated thyroid results in an increase in protease activity and a decrease in arginase activity of submaxillary gland extracts. Hypophysectomy or the administration of propylthiouracil increases arginase activity of submaxillary gland extracts.

3. The protease activity per gram of submaxillary gland tissue in pilocarpine-stimulated animals does not change over an 8-hour period although there is a decrease in the wet weight of the gland. The average diameter of the submaxillary granular tubules, presumably the site of the protease, does not change following pilocarpine stimulation.

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FLUORIDE CONTENT OF HUMAN TEETH FROM THE ORIENT AND THE CANADIAN ARCTIC

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THE ENAMEL and dentin of residents of Delhi and Bombay, India, have been shown by Shaw, Gupta, and Meyer[1] to contain high levels of fluoride in comparison to the fluoride levels in enamel and dentin from low-fluoride areas in the United States.[1, 2] The present survey was undertaken to determine the fluoride levels of enamel and dentin from other population groups in the Orient and from Eskimos and Indians in the Canadian Arctic areas.

EXPERIMENTAL

During the course of this survey, teeth were obtained from a total of 534 individuals in the United States, India, Pakistan, Iran, and the Canadian Arctic. Extracted teeth were available from 15 patients in Boston, Mass., whose ages varied from 9 to 51; most of these patients were in the 14- to 25-year age group. The East Indian teeth were obtained from 5 widely separated areas: from 67 patients in Delhi, whose ages varied from 10 to 65, mainly from the 25- to 50-year age group; from 52 patients in Calcutta, whose ages varied from 12 to 62; from 23 patients in Bombay, whose ages varied from 20 to 64; from 29 patients in Lucknow of unknown age; and from 22 patients in Dibrugarh, which is located in Assam, whose ages varied from 18 to 60. Teeth from 15 Pakistani military personnel stationed in Lahore and from 6 Iranians, natives at an army base, were obtained by a United States Public Health survey team. The Canadian samples were from the Eastern and Western Arctic regions and Northern Alberta, and were classified into 5 groups on the basis of dietary and racial backgrounds. The first group consisted of 197 Eskimos who currently were living along the coast under primitive, isolated circumstances and were consuming predominantly a seal and fish diet. In the second group were 6 Eskimos of the nomadic caribou culture who lived inland and followed the caribou herds and whose diet was largely composed of meat, berries, etc., with a minimum amount of the refined foods associated with civilization. The third, fourth, and fifth groups were composed, respectively, of 32 Eskimos who currently were living in the vicinity of trading posts and were consuming a diet composed to a large extent of foods purchased at the posts, 13 Indians on a primitive diet living in remote areas, and 57 Indians who currently

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TABLE I
FLUORIDE CONTENT OF HUMAN TEETH

LOCATION	NUMBER OF INDIVIDUALS	NUMBER OF SAMPLES	FLUORIDE CONTENT* (ppm)	CRITICAL RATIO†	NUMBER OF SAMPLES	FLUORIDE CONTENT* (ppm)	CRITICAL RATIO†
UNITED STATES							
Boston	15	12	54 ± 7 (20-125)	5.0	11	86 ± 15 (28-241)	6.4
INDIA							
Delhi	67	61	261 ± 21 (61-967)	3.2	66	730 ± 50 (164-1982)	4.9
Calcutta	52	50	145 ± 10 (47-343)	2.9	51	375 ± 25 (71-820)	2.4
Bombay	23	22	171 ± 18 (57-351)	6.1	22	423 ± 38 (130-771)	7.4
Lucknow	29	28	190 ± 13 (93-399)	2.8	29	589 ± 37 (193-966)	4.8
Dibrugarh	22	21	112 ± 13 (45-245)	4.2	22	278 ± 35 (122-750)	5.7
PAKISTAN							
Lahore	15	15	204 ± 20 (84-334)		15	627 ± 61 (286-1063)	
IRAN							
Army base	6	6	267 ± 58 (77-519)		6	911 ± 160 (298-1394)	
CANADIAN ARCTIC							
Eskimo—primitive, coastal culture	197	172	164 ± 7 (26-441)	3.8	195	515 ± 19 (67-1630)	5.3
Eskimo—primitive, caribou culture	6	6	172 ± 50 (64-346)		6	471 ± 105 (177-850)	
Eskimo—trading post residents	32	28	185 ± 19 (23-460)	3.1	32	596 ± 64 (36-1321)	4.0
Indians—primitive	13	13	181 ± 30 (75-389)	2.0	13	478 ± 51 (163-851)	2.8
Indians—trading post residents	57	52	118 ± 10 (37-397)		57	309 ± 34 (60-1235)	

*The values contained in parentheses under each average are the extremes noted in each population group.

†Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the differences between the means. The two lines from each critical ratio point to the two means that are being compared. Whenever the critical ratio is less than 2.0, the difference between the means is considered statistically to be insignificant; when from 2.0 to 2.9, borderline significance; when 3.0 or higher, highly significant.

were living in settlements and villages where commercially prepared foods were available. Ages, location of childhood residence, and other comparable data were to a large extent unavailable for the Eskimo and Indian population groups.

After detailed visual observation and classification according to types, each tooth was carefully cleaned of all calculus, carious material, and superficial surface stains. The crown was then cut from the root or roots by a power jig saw and pulverized in a diamond mortar until the particles were sufficiently small to pass through a 100-mesh sieve. A flotation-centrifugation procedure, using a bromoform-acetone mixture, as described by Manly and Hodge,[3] was used to separate the enamel from the dentin. Distillation of the fluoride in the enamel and dentin samples from contaminating materials was conducted in polyethylene bottles by the procedure described by Singer and Armstrong.[4] The colorimetric method described by Icken and Blank[5] was used to determine the amount of fluoride in the distillate.

RESULTS AND DISCUSSION

The results of the fluoride analyses are presented in Table I. Samples of the enamel and dentin from Boston teeth were found to have the lowest levels of fluoride, 54 ppm and 86 ppm of fluoride, respectively. All of the other population groups had levels of fluoride in the enamel and dentin that were judged statistically to be significantly higher than the levels in the Boston teeth. The small group of Iranian teeth had the highest levels, with averages of 267 ppm of fluoride in the enamel and 911 ppm of fluoride in the dentin. The Delhi teeth had the next highest average values, with 261 ppm of fluoride in the enamel and 730 ppm of fluoride in the dentin. These average values were very similar to the average values reported earlier for other groups of teeth collected in Delhi.[1] The present average values for Delhi teeth were significantly greater than the average values for the enamel and dentin from the other 4 areas of India, which ranged from a high of 190 ppm of fluoride for enamel and 589 ppm of fluoride for dentin for the Lucknow teeth to a low of 108 ppm of fluoride for enamel and 278 ppm of fluoride for dentin for the teeth from the individuals in Dibrugarh. The average values for Bombay teeth were again closely similar to the average values for the earlier sample of Bombay teeth for which values have been reported.[1] The differences between the levels of fluoride for the teeth from individuals in Bombay, Calcutta, and Lucknow were found to be statistically insignificant. However, the differences between the levels for these 3 areas and the levels for the teeth from Dibrugarh were statistically of borderline significance. Generally, the differences in the levels of fluoride in dentin were of greater statistical significance than similar differences in the levels of fluoride in enamel. This trend was especially true in the comparison of the levels of fluoride in the Lucknow and Calcutta dentin.

The Pakistani teeth were found to have levels of 204 ppm of fluoride for enamel and 627 ppm of fluoride for dentin, both appreciably lower than the

Delhi values, but these differences were not sufficiently large, in view of the large standard errors of the means, to be of statistical significance.

An evaluation of the causes of extraction for teeth of the Indian citizens revealed that about 40 per cent of the Delhi teeth were extracted for caries and 55 per cent for various types of periodontal disease. On the other hand, caries was given as the reason for the extraction of teeth in Bombay in only a very few cases, whereas periodontal disease was given as the reason for 60 per cent of the extractions in Calcutta, where caries caused 25 per cent. The teeth from individuals in Dibrugarh were extracted mainly for periodontal disease (80 per cent) and only secondarily for caries (12 per cent). Similar data for the Lucknow teeth were not available. Since these samples were relatively small, the distribution of causes for extraction among dental caries, periodontal disease, and other reasons is not to be considered as a valid index of the relative prevalence of oral disease in these areas. Further evaluation on the relationship between fluoride levels and caries for the Delhi teeth revealed that those teeth which had been extracted for caries had slightly lower levels of fluoride than teeth extracted for other reasons. However, this difference was not statistically significant.

The average levels of fluoride for the Canadian enamel and dentin samples were in the same range as those of the Indian samples, excluding Delhi. The fluoride levels for the enamel and dentin samples for the various Canadian groups differed from each other by relatively small amounts except for the group of Indians living in close contact with civilization. The levels of fluoride in the enamel and dentin of the coastal Eskimos on a primitive fish diet and of the Eskimos living in close proximity to trading posts were significantly higher than that of the Indians on a civilized diet. There was no significant difference between the levels of fluoride in the enamel and dentin of the 3 groups of Eskimos, two of which were currently consuming primitive diets, and the third a diet composed of large amounts of commercially available foods. However, all 5 of the Eskimo and Indian groups had levels of fluoride in their enamel and dentin that were significantly higher than the fluoride levels of the teeth collected in Boston.

The Eskimos and Indians living in the Canadian Arctic on civilized diets generally had more caries than their contemporaries on primitive diets. Whereas 60 per cent of the teeth extracted from Eskimos on primitive diets were lost because of periodontal disease, most of those on a civilized diet had teeth removed for caries. This was also true for the Canadian Indians, although the Indians on the primitive diet had a higher percentage of extractions because of caries than Eskimos on primitive diets. The Eskimos on primitive diets whose teeth had been extracted because of carious lesions had slightly lower levels of fluoride in both enamel and dentin than their contemporaries whose teeth had been lost by reason of periodontal disease and other causes. As in the teeth from Delhi, this difference was statistically insignificant.

Water samples were not available in this survey. In many cases, no water samples would have been representative for the individuals whose teeth were

available for analyses. This comment is particularly pertinent for individuals in Iran and Pakistan who were military personnel stationed at bases in those countries and who were undoubtedly from widely separated areas of their countries. Again, in the case of the Eskimos and Indians in the Canadian Arctic areas, communal water supplies were not available and widely different sources of water undoubtedly were used in the course of their lifetimes. To a lesser extent, similar problems arise in connection with the population groups from India where exact knowledge of the water supplies available during childhood may not be obtainable by reason of change in residence, installation of communal water supplies, and such other factors as seasonal variations in the available water.

The variations in fluoride values within each of these population groups is large enough to cause speculation as to the reason. Variations in fluoride intake as well as variations in individual utilization of ingested fluoride may be involved. In each population group sampled in India, Pakistan, Iran, and the Canadian Arctic areas, the lowest fluoride values in the enamel are as low or almost as low as the average value for fluoride in the enamel of the teeth calcified during residence in Boston where fluoride ingestion is known to be very low. To a lesser extent, this observation can be duplicated with respect to the low levels of fluoride in the dentin of the various population groups. In every population group, there were from one to several enamel fluoride values that were lower than the highest values for enamel fluoride observed in the teeth extracted in Boston. With respect to the dentin from every population group except the Pakistani and Iranian groups, anywhere from one to several samples were analyzed that had lower fluoride concentrations than the highest values for dentin in teeth extracted in Boston. The extent of overlapping was remarkable in view of the large differences in the average values for the various population groups.

Except for the teeth from Boston, the average fluoride levels for the enamel and dentin from the other population groups were sufficiently high to give a pronounced resistance to dental caries if all other associated factors of oral environment, tooth structure, oral hygiene, etc., were equivalent.

Obviously, this survey represents only cursory evaluations of various populations in several different areas of the world, and the results cannot be broadly applied to the populations in those areas. However, the composite nature of a number of these groups and the close comparison of the new averages for teeth from Delhi and Bombay with earlier averages strongly suggest that relatively high fluoride values occur in the enamel and dentin in widespread areas of the world where low susceptibility to dental caries has been reported previously. There is a real need for specific information on the source of the fluoride and the amounts of fluoride ingested by populations with these types of backgrounds. Collection of information of this nature can be done only in surveys conducted with the greatest care in the actual areas where the citizens' teeth are under investigation.

SUMMARY

Fluoride determinations have been made on the enamel and dentin of teeth from 534 individuals from the United States, Pakistan, Iran, 5 areas of India, and 5 racial and cultural groups in the Canadian Arctic regions.

In comparison with the average fluoride levels of enamel and dentin from teeth extracted in Boston, the average fluoride levels of enamel and dentin from each of the other 12 population groups were significantly higher. The highest levels of fluoride were observed in teeth extracted from military personnel in an Iranian army base and from residents of Delhi, India. The lowest levels of fluoride other than in teeth from Boston were observed in a group of Indians living in the Canadian Arctic in close proximity to trading posts or in other settlements and in residents in Dibrugarh, India.

These data suggest that relatively wide areas of the world where low dental caries susceptibility prevails may provide circumstances whereby high levels of fluoride may be available during the formation of the teeth.

We are indebted to Dr. Walter C. Guralnick, Boston, Mass.; Dr. U. S. Mallick, Calcutta Dental College, Calcutta, India; Dr. T. N. Chawla, Lucknow Dental College, Lucknow, India; Dr. C. Vilasiny, Medical College, Dibrugarh, India; Dr. P. C. Jayna, Chandi Chowk, Delhi, India; Dr. S. S. Khera, Sir C. E. M. Dental College, Bombay, India; Dr. A. E. Schaefer, Interdepartmental Committee on Nutrition for National Defense, Department of Health, Education and Welfare, Bethesda, Md.; Dr. Om P. Gupta, New York University College of Dentistry, New York, N. Y.; Dr. C. H. Carley, Charles Camsell Hospital, Edmonton, Alberta, Canada; and Dr. J. H. Wiebe, Indian and Northern Health Services, Department of National Health and Welfare, Ottawa, Ontario, Canada, for willingness to collect personally or make arrangements for their staff personnel to collect the teeth used in these studies and the vital statistics of the donors.

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VARIABILITY OF TOOTH FORMATION

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INTRODUCTION

DATA on the variability of tooth formation are of potential use in a wide variety of applications. With juvenile fossil "ancestors" of man, teeth alone may have to serve as indicators of age.[1] The identification of skeletal remains, cadavers, or amnesia victims may be speeded by good age-estimates utilizing the teeth.[2] Where endocrinopathy is known or suspected, comparison of the dental status of individuals with appropriate norms may be of potential diagnostic value, and may help to elucidate the effects of endocrine secretions on tooth development.[3, 4, 5] It is useful, therefore, to have adequate information on the "normal range" of variability of tooth formation, using either the $\pm 2\ \sigma$ limits currently accepted[6] or other limits, precisely defined.

The literature prior to 1930 provides little assistance in this task. Although there is no lack of tables, the accuracy of the early estimates is open to question, and ranges of variability are almost totally lacking. The tables of Pierce,[7] Black,[8] and Legros and Magitot[9, 10] simply do not agree with clinical experience. The atlas of Remington, and the trilingual photographic atlas of Witzel[11] are beautifully printed, but inadequate, as is the tabulation reprinted by Churchill in 1933.[12]

Most contemporary references to tooth formation and the variability of tooth formation are derived from the studies of Logan and Kronfeld,[13] and the table published by Kronfeld in 1935.[14] Arey's table, as printed in *Developmental Anatomy* (ed. 6, 1957[15]) is clearly modified from Kronfeld's although not so designated. Wilkins attributes his Table VI (Ref. 16, p. 38) to Holt and McIntosh,[17] and they (via Schour and Massler[18]) to Kronfeld.[14] The "norms" for tooth formation in *The Handbook of Biological Data*[6] are evidently derived from Kronfeld[14] through Schour and Massler. Thus, any worker who wishes information on the variability of tooth formation knowingly or unknowingly makes use of the Logan-Kronfeld data and the Kronfeld values.

However, in the course of our longitudinal investigations on the development of the secondary dentition, we encountered increasing evidence that the Logan-Kronfeld ranges were unduly narrow. Many children in our series fell outside of the published ranges, occasionally by as much as 3 to 4 years. Accordingly, we prepared new estimates of variability for 3 stages of 5 posterior teeth, and found a much larger range of variability than had been

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claimed hitherto. Yet, the new, larger estimates of variability were not larger than estimates of variability for other age-comparable developmental phenomena.

The present paper, then, is directed to two ends. First, it is our purpose to provide new and improved estimates of variability for 3 stages of formation of the mandibular premolar and molar teeth. Second, it is our intention to contrast the variability of formation of these teeth with other measures of osseous and sexual development, using, for the most part, data derived from the same population sample for strictest comparability.

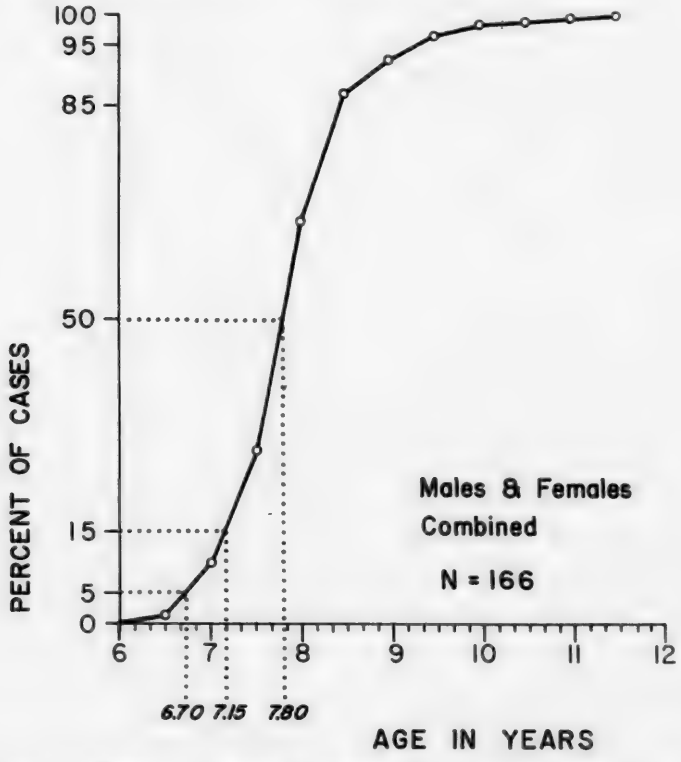

Fig. 1.—Cumulative percentile distribution for crown completion, root formation of $\overline{P}_2$ showing location of 5th, 15th, and 50th percentiles by interpolation. Although interpolations made by five different observers agree to within ± 0.03 years, the final values used in this study are rounded off to the nearest 0.1 years (see Table I).

METHODS AND MATERIALS

The present study is based upon serial longitudinal oblique-jaw x-rays, supplemented by serial lateral-skull plates of 255 white Ohio-born children of Northwest European ancestry. As a group, the boys and girls were clinically healthy, primarily of the middle socioeconomic class, and relatively free from excessive decay and premature loss of dm_1, dm_2, and M_1. Multiple births and children with growth failures or developmental defects were excluded from the tabulations.

Three widely-spaced stages of tooth formation, as discernible in the radiographs, were selected to correspond as nearly as possible with the "stages" given in the literature. The stages employed and previously described by us include (a) beginning calcification,* (b) beginning root formation, and (c) apical closure,[19, 20] as shown in Figs. 2-4.

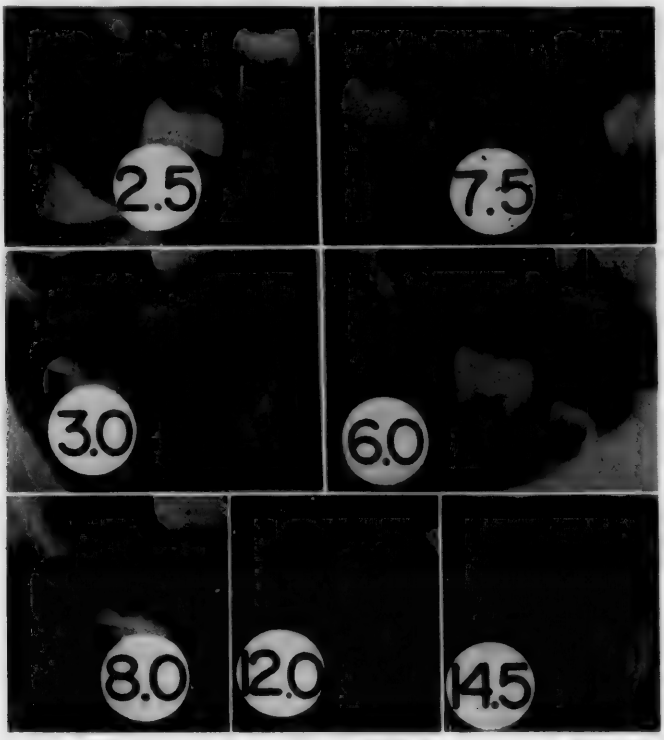

Fig. 2.—Extremes in beginning calcification of the mandibular premolar and molar teeth. *Above*, early beginning calcification of P_2 at 2.5 years (Case No. 416), late beginning calcification of P_2 at 7.5 years (Case No. 333). *Middle*, extremes in beginning calcification of M_2 (Cases 223 and 371). *Below*, fairly early beginning calcification of M_3 (Case No. 299), and late beginning calcification of M_3 in Case No. 67, as shown at 12.0 and 14.5 years.

In making the statistical analyses, the possibility of secular changes was ruled out.[19] Since skewness could not be excluded in advance, the 5th, 15th, 50th, 85th, and 95th percentiles were determined, usually by interpolation from the cumulative frequency curves (Fig. 1). The quartiles (Q_1 and Q_3) were also determined, in order to make use of the semiquartile test of skewness.[21] In addition, the standard deviation(σ) and its derivative, the coefficient of variation (CV) were computed in order to compare the relative variabilities of the different developmental stages of the 5 mandibular molar and premolar teeth.

*Although calcification is histologically demonstrable before it is evident on radiographs, no attempt was made in the present study to adjust the radiographic data systematically, since such a procedure would not affect the *ranges*. Moreover, it was felt that data on calcification as seen in x-rays would be more useful clinically than data that attempted to simulate histologic findings.

Since the coefficient of variation of the first stages of the early-forming teeth was unrealistically high, due to the fact that birth is ordinarily assigned zero time, all of the coefficients of variation given in the present study were adjusted to conception. A preliminary test on M_1 and P_1, using individually-computed gestation lengths for 90 to 100 individuals, showed that a group correction of 0.75 years yielded essentially similar results. Accordingly, the group correction was then employed throughout.*

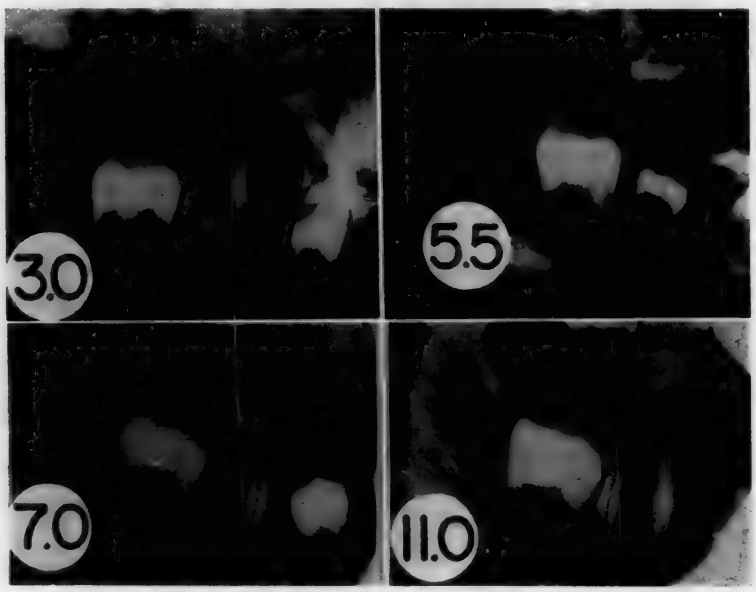

Fig. 3.—Extremes in beginning root formation for M_1 (Cases 220, age 3.0 years and 371, age 5.5 years), and M_2 (Cases 356, age 7.0 years and 280, age 11.0 years). These extreme values greatly exceed the ranges given in the literature (see text).

Due to the fact that the children in the Fels Longitudinal Studies are part of a longitudinal program with continuing recruitment,[21] there are necessarily more data for the earlier stages of the earlier-forming teeth than for the later stages of the later-forming teeth. Again, since some of the children were in the latter part of their first decade at the time the present radiographic series was begun (1939), not all of the data refer to the same subsample. Therefore, the number of children involved in each tabulation is shown separately.

Although the present data are derived from a clinically healthy and fairly sizeable sample of American-born white children, the tabulations are not intended to serve as norms, for the samples are, in most cases, inadequate for that purpose. We trust that workers who refer to the present findings will bear this precautionary statement in mind.

*For beginning calcification of $\overline{P}_1$, the combined-sex individually conception-corrected CV was 11.63, as compared to a CV of 11.55, using the group correction. For $\overline{M}_1$, the comparable values were 4.83 and 5.10. N was 99 and 98, respectively.

FINDINGS

To begin with, percentiles were computed for each of the 3 developmental stages (beginning calcification, beginning root formation, and apical closure) of the 5 mandibular teeth considered, and for boys and girls, respectively.

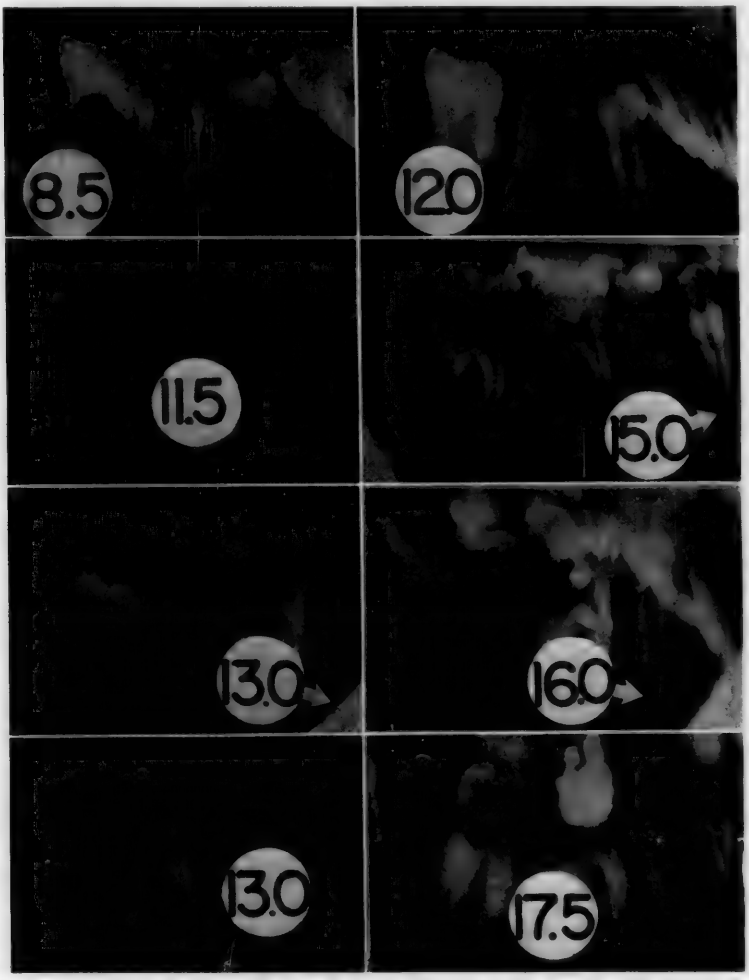

Fig. 4.—Examples of early and late apical closure: M_1 8.5-12.0 years (Cases 220F and 190M); P_1, 11.5-15.0 years (Cases 87M and 207M); and P_2, 13.0-16.0 years (Cases 263F and 207M). Examples given for M_2 include Cases 240M (13.0 years) and 157M (17.5 years).

Although skewness was minimal for most of the 28 sex-specific distributions, as measured by the semiquartile test of skewness,[22] moderate to high degrees of skewness were observed for a few distributions. Accordingly, the assumption of normality of distribution was not made, and values corresponding to the 5th, 15th, 50th, 85th, and 95th percentiles are provided in the tabulations (Table I). For practical purposes, the 15th-85th percentile values given here may be taken as equivalent to the $\pm 1\ \sigma$ limits.

TABLE I. VARIABILITY OF TOOTH FORMATION

TOOTH	BOYS								GIRLS								COMBINED SEX							
	NO.	PERCENTILES (YR.)					CV_1*	CV_2†	NO.	PERCENTILES (YR.)					CV_1*	CV_2†	NO.	PERCENTILES (YR.)					CV_1*	CV_2†
		5TH	15TH	50TH	85TH	95TH				5TH	15TH	50TH	85TH	95TH				5TH	15TH	50TH	85TH	95TH		
Beginning Calcification																								
P_1	98	1.9	2.1	2.4	2.9	3.1	13	10	66	1.6	1.7	2.1	2.4	2.8	15	11	164	1.6	1.9	2.3	2.7	3.0	15	12
P_2	100	2.8	3.0	3.4	3.9	4.4	17	14	79	2.6	2.9	3.4	4.3	4.8	16	14	179	2.7	3.0	3.4	4.0	4.7	17	14
M_1	98	0.09	0.10	0.16	0.23	0.24	12	3	59	0.02	0.06	0.14	0.21	0.23	33	7	157	0.04	0.09	0.16	0.22	0.24	22	5
M_2	113	3.0	3.1	3.6	4.3	5.2	18	15	83	2.7	3.0	3.5	4.3	4.6	14	12	196	2.8	3.1	3.5	4.3	4.8	16	14
M_3	69	7.4	8.1	9.1	10.4	11.0	12	11	66	7.6	8.3	9.0	10.4	11.4	13	12	135	7.5	8.1	9.0	10.3	10.9	12	11
Beginning Root Formation																								
P_1	97	6.1	6.6	7.1	7.9	8.2	8	7	75	6.0	6.4	6.9	7.6	7.9	8	7	172	6.0	6.5	7.0	7.8	8.1	8	7
P_2	90	6.9	7.3	7.9	8.5	9.4	9	9	76	6.6	7.0	7.7	8.5	9.1	9	8	166	6.7	7.2	7.8	8.5	9.3	9	8
M_1	105	3.2	3.5	4.0	4.3	4.9	11	9	70	2.9	3.1	3.7	4.4	4.7	14	11	175	3.1	3.3	3.9	4.5	4.8	12	11
M_2	89	7.6	7.9	8.5	9.6	10.4	11	10	88	7.1	7.6	8.4	9.4	10.2	12	11	177	7.3	7.7	8.4	9.5	10.2	11	10
M_3	34	12.4	13.2	14.2	15.5	16.6	9	8	19	11.8	12.9	14.9	16.9	17.3	12	11	53	12.0	13.1	14.6	16.3	17.1	10	9
Apical Closure																								
P_1	24	11.3	12.1	12.5	13.7	14.0	7	6	16	11.1	11.4	12.2	12.9	14.1	7	6	40	11.2	11.6	12.4	13.6	14.0	7	7
P_2	18	12.2	12.5	13.7	15.1	15.5	8	7	14	12.1	12.2	13.1	14.0	14.6	6	6	32	12.1	12.3	13.4	14.8	15.4	7	7
M_1	48	8.7	9.1	10.1	10.9	11.7	8	7	39	9.0	9.6	10.4	11.2	11.4	7	7	87	8.8	9.3	10.2	11.1	11.6	8	7
M_2	22	12.7	13.2	14.9	16.7	17.7	9	8	15	13.1	13.3	13.9	16.2	17.1	8	8	37	12.8	13.3	14.6	15.9	17.6	9	8

*$CV_1 \frac{100\ \sigma}{\bar{X}}$ using age since birth.

†$CV_2 \frac{100\ \sigma}{\bar{X}}$ using age since conception (see text).

As might be expected, variability of tooth formation increased steadily with advancing median age. Thus, the early developmental stages of early-forming teeth were notably less variable than the later developmental stages of the later-forming teeth. Over-all, the correlation between one measure of variability (the 15th-85th percentile range) and the median age-for-stage was nearly perfect, with an obtained r of 0.96. The statement that variability (15th-85th percentiles) approximates 28 per cent of the median age for any stage of any tooth adequately fits the data. By this token, the percentagewise *variability* of M_3, for example, is of the same order of magnitude as that for the other teeth.

Although the median age for a given developmental stage of a particular tooth proved slightly higher in the boys, as previously noted,[19] there was no consistent sex difference in variability, as may be seen in Table I. Variability in the male exceeded that in the female as often as the reverse was true. Thus, by this simple sign test, as by the more exact F test,[23] it could not be stated that one sex was more variable than the other, in the formation of the teeth here considered.

Throughout, *combined-sex* values for tooth formation were obtained by pooling all data, regardless of sex (not by averaging male and female percentiles). Properly, such combined-sex distributions ought not to be effected, since there is a true sex difference in the times of beginning calcification, beginning root formation, and apical closure. However, such combined-sex distributions were necessitated by the fact that all previously published "normative" values were not sex-specific, and one purpose of the present investigation was to make comparisons with previous tabulations. Further, the combined-sex percentiles given in Table I have the advantage of being based on the largest possible sample, 150-200 cases in 8 of the 14 relevant individual distributions.

Using the combined-sex values, as given in Table I, it was then possible to make comparisons with previously published data. Two difficulties were immediately encountered. Except for one secondary reference, *The Handbook of Biological Data*,[6] which specified the $\pm 2\ \sigma$ limits, none of the authors explicitly defined the "ranges" as given by them. Again, there was confusion in the literature as to the source of the data given in the various tabulations. We have, therefore, adopted the conservative plan of giving our 5th-95th percentile limits for comparison, and of grouping authorities according to the values reported by them. Thus, Holt and McIntosh,[17] and Wilkins[16] are listed with Kronfeld,[14] since the values given by all three authors are identical. Arey[15] is grouped with Schour and Massler[18] (who combined maxillary and mandibular ranges), while Spector's data in the *Handbook of Biological Data*[6] are presented separately.

As is immediately apparent in Table II, where data on the variability of tooth formation are compared, the ranges obtained in the present study of dental development greatly exceed those in the literature. Although the true "range" should, of necessity, exceed our 5th-95th percentile values, the "ranges" given by Kronfeld, Holt and McIntosh, Wilkins, Schour and Massler, Arey, and Spector fall quite short of ours, usually by a year, and often

more. For beginning calcification of P_2, beginning calcification of M_1, and beginning root formation of P_2, the discrepancy approximates or exceeds two years. Moreover, the previous ranges of variability for apical closure (root completion) are both narrower than ours, and inexplicably uniform from P_1—M_2, whereas ours increase progressively from M_1—M_3, in accordance with the general picture of increasing variability.

TABLE II
VARIABILITY OF $\overline{P_1}$—$\overline{M_3}$ AS GIVEN BY VARIOUS AUTHORS

MANDIBULAR TOOTH	LOGAN AND KRONFELD[13]	KRONFELD,[14] HOLT AND MC INTOSH,[17] WILKINS[16]	SCHOUR AND MASSLER,[18] AREY[15]	SPECTOR[6]	THIS STUDY (PERCENTILES 5TH – 95TH)
		Beginning Calcification (Years)*			
P_1	1.50 – 2.00	1.75 – 2.00	1.50 – 2.00	1.75	1.6 – 3.0
P_2	2.00 – 2.50	2.25 – 2.50	2.00 – 2.50	2.50†	2.7 – 4.7
M_1	0.08 – 0.33	Birth	Birth	Birth	0.04 – 0.24
M_2	2.00 – 2.50	2.50 – 3.90	2.50 – 3.00	2.75	2.8 – 4.8
M_3	7.00 – 9.00	8.00 – 10.00	7.00 – 10.00	7.00 – 10.00	7.5 – 10.9
		Crown Completion—Root Formation‡ (Years)			
P_1	----	5.00 – 6.00	5.00 – 6.00	----	6.0 – 8.1
P_2	----	6.00 – 7.00	6.00 – 7.00	----	6.7 – 9.3
M_1	----	2.50 – 3.00	2.50 – 3.00	----	3.1 – 4.9
M_2	----	7.00 – 8.00	7.00 – 8.00	----	7.3 – 10.2
M_3	----	12.00 – 16.00	12.00 – 16.00	----	12.0 – 17.1
		Root Completion—Apical Closure§ (Years)			
P_1	----	12.00 – 13.00	12.00 – 13.00	12.00 – 13.00	11.2 – 14.0
P_2	----	13.00 – 14.00	12.00 – 14.00	12.00 – 14.00	12.1 – 15.4
M_1	----	9.00 – 10.00	9.00 – 10.00	9.00 – 10.00	8.8 – 11.6
M_2	----	14.00 – 15.00	14.00 – 16.00	14.00 – 16.00	12.8 – 17.6
M_3	----	18.00 – 25.00	18.00 – 25.00	18.00 – 25.00	Under 18 to over 26

*Defined as "time of beginning calcification,"[23] "hard tissue formation,"[14] "first evidence of calcification,"[16, 17] "apposition of enamel and dentin,"[18] "calcification of crown begins."[15]
†Probably an averaging error.
‡Designated "enamel completed" by Kronfeld,[14] "crown completed" by remaining authors.
§"Root completed," [14, 16–18] "calcification of root ends."[15]

The difference between the ranges given here and those in the literature, quite apparent in Table II, is much more dramatic when graphed, as in Fig. 5. Using the Kronfeld table for comparison (since mandibular teeth are not combined with maxillary teeth, as in Schour and Massler[18] and Arey[15]), the ranges of variability as given in the literature are approximately one third as great as ours. This generalization holds true not only for beginning calcification, where differences in the investigative criteria may be at least partially responsible, but also for apical closure.

In short, the data from the present study, based on 255 children, and with 30-190 examples of a given stage for a particular tooth, suggest that the true variability of tooth formation is approximately 3 times as great as has been accepted so far.

Finally, since tooth formation appeared to be at least 3 times as variable as had been claimed hitherto, it seemed pertinent to compare the variability of tooth formation with that of other commonly measured developmental phenomena. Accordingly, the data from Table I were compared with data on

7 age-associated phenomena, selecting a variety of criteria that would cover the rather wide age-range involved. These developmental variables included (a) eruption of the deciduous[24] and permanent teeth,[25] (b) time of appearance of the first 61 centers of ossification,[26] (c) "wrist age,"[27] (d) age at menarche, (e) age at beginning breast development,[28] (f) age at beginning of pubic hair development,[28, 29] and (g) age at beginning of enlargement of the genitalia.[29] Except for permanent tooth eruption, based on Hurme's norms,[25] and wrist age (Greulich and Pyle[27]), all data on variability were derived from and therefore uniquely applicable to the present population sample.

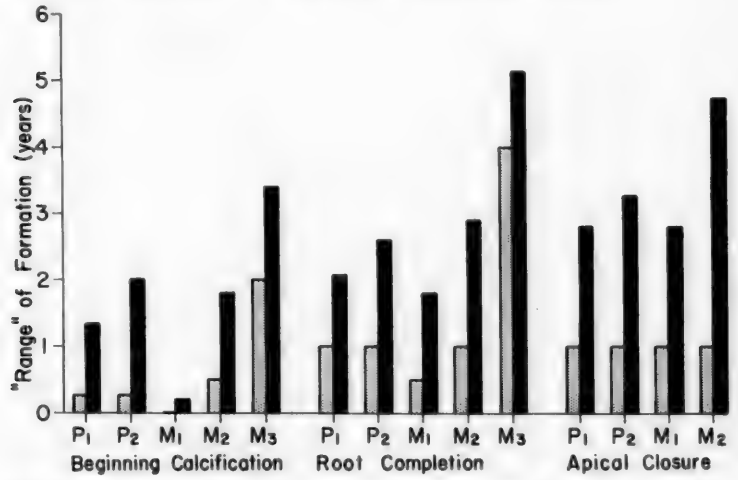

Fig. 5.—Comparison of the "ranges" for several stages of formation of P_1—M_3, as given by Kronfeld (*stippled bars*) and as determined in the present study (*solid bars*). Clearly the variability of these teeth has been considerably underestimated hitherto.

However, two statistical problems had to be met. The first was the need for a measure of *relative* variability, since developmental phenomena covering an 18-year range had to be compared. Despite our initial reluctance to do so, because of the skewness of some tooth-formation curves, the familiar coefficient of variation (CV) was finally adopted as a practical necessity. Again, there was need to correct all values to conception, rather than to birth, since this latter procedure resulted in spuriously high values of the coefficient of variation for such early phenomena as beginning formation of M_1 and P_1, time of appearance of the distal epiphysis of the radius, etc. Accordingly, and as described in the previous section, a *group* correction of 0.75 years was added to all values, since it had been demonstrated that individual corrections for length of gestation did not yield appreciably different results.

In essence, tooth formation proved no more variable, and often less variable, than the other age-correlated developmental variables. For beginning calcification of P_1—M_3, the combined-sex conception-corrected CV ranged from 5.1 to 13.9, with a mean (for 5 teeth) of 11.1. In contrast, the comparable mean conception-corrected combined-sex CV for the first 61 ossification centers exceeded 19, that for 8 selected centers of ossification (in boys) exceeded 19,

and the approximate conception-corrected combined-sex CV for wrist age (Greulich and Pyle[27]) during the first 8 years of life was 15. Tooth formation (mean CV of 11) and deciduous tooth eruption (mean CV approximately 15) both proved less variable than osseous development.

With beginning root formation, the mean conception-corrected combined-sex coefficient of variation was 9.2 (range 7.3 to 10.6) for 5 teeth, a value obviously below that for skeletal age, but comparable to the combined-sex and to the sex-specific CV of 12 as computed from Hurme's norms, again demonstrating that the variability of tooth formation is by no means greater than that of other developmental "events." Moreover, it suggested that the teeth are possibly less variable in all developmental stages through to eruption.

With apical closure, the mean conception-corrected combined-sex CV was 7.2. Here, however, sex-specific values were of more use, since the various criteria selected for comparison (menarche, breast development, etc.) did not allow meaningful combined-sex values. Taking a mean sex-specific coefficient of variation of 7 for apical closure, comparative estimates of variability were 9.4 (pubic hair stage I, girls; pubic hair stage II, boys), 8.5 (menarche), 7.4 (genitalia stage II, boys), and 9.9 (beginning breast formation, girls). Thus, the variability of the final stage of tooth formation, namely, apical closure, was of the same order of magnitude as the variability of other developmental phenomena also occurring on the average between the tenth and the fourteenth year.

In summary, tooth formation proved to be no more variable than other developmental phenomena, and, in comparison to osseous maturation, it was less. The rather close similarity between the variabilities for apical closure P_1—M_2, and sexual maturation, may possibly be due to a communality of responses to the particular steroid hormones involved. Even more important, they indicate that the present estimates of variability of tooth formation are reasonable.

DISCUSSION

It is evident from the present data that tooth formation is a more variable developmental phenomenon than has been realized to date. Using a sizeable sample of clinically healthy white children, and up to 196 examples of a particular formation stage for a given tooth, the 5th to 95th percentile ranges for the posterior mandibular teeth are, on the average, 3 times as great as the "ranges" given in the literature. Expressing relative variability by the coefficient of variation (CV), the conception-corrected combined-sex CV averages 11 per cent for beginning calcification, 9 per cent for beginning root formation, and 7 per cent for apical closure. In all probability the anterior teeth and the maxillary dentition would similarly prove more variable than the customary tables have led us to assume.

Notwithstanding the greater variability exhibited in the present data, tooth formation is not more variable, and often less variable than other developmental measures. As compared to an average conception-corrected coefficient of variation of 11 for beginning calcification of P_1—M_3, the mean CV

for "skeletal age"[27] is 19, that for the time of appearance of 61 individual ossification centers[26] is 20. Similarly, while the mean conception-corrected CV for apical closure of P_1—M_2 is 7, the comparable mean CV for 4 measures of sexual maturation[28, 29] is 8.3. On a uniform basis, using only maturational data derived from the present population sample, tooth formation would appear to be approximately half as variable as other developmental phenomena. In consequence, tooth formation may be valuable in providing age-estimates for skeletalized material and unknown cadavers ranging from infancy to adolescence.

Although, it is commonly assumed that such phenomena as tooth eruption stages are symmetrical and normal in distribution, this assumption does not hold for many of the stages. While it is possible that some of the skewness in the present data is due to sampling error and to the class-intervals necessarily used, the fact remains that the standard deviation (σ) would seriously overestimate the lower percentile values for beginning formation of P_2. For the present, therefore, it is prudent to give percentiles rather than means and standard deviations for tooth formation.

Inevitably, there is reason to wonder why the present ranges so greatly exceed those published and widely reprinted by Kronfeld[14] and Logan and Kronfeld.[13] It is true, of course, that "normative" values are frequently amended as more data accumulates; norms for tooth eruption are a striking case in point. Rarely, however, is it necessary to alter ranges by a factor of two or three. After critical analysis of the original publications, several possible explanations suggest themselves and merit consideration here.

It is possible that data derived from histologic sections are not at all comparable to roentgenographic findings, unless silver staining is used.[30] This objection would be most applicable to beginning calcification, and least applicable to apical completion. Nevertheless, the discrepancies apply equally to beginning calcification, beginning root formation, and to apical closure, the last presumably derived from roentgenograms throughout. Moreover, it is ranges, and not mean or median values, that are under comparison (vide supra).

Again, it is possible that the "ranges" are differently defined. Neither Logan and Kronfeld,[13] nor Kronfeld[14] are explicit on this matter, merely suggesting (at one point) that their values are "relative." But if their ranges truly cover the "extreme range," our 5th-95th percentile ranges should fall far *short* of theirs. The converse is the case, however, as seen in Fig. 5, and especially in Figs. 2 to 4.

Third, it is possible that the unique sample investigated by Logan and Kronfeld is responsible for the discrepancies. "In most of the cases studied," they write, "the child died of tuberculosis or associated disease. . . . Some died of severe intestinal disturbances. . . . Several of the young children . . . apparently died of debility."[13] At least 20 per cent of the Logan and Kronfeld sample were developmentally defective. Such a sample might be characterized by reduced variability of tooth formation, although our own studies fail to support this idea.

To our minds, the most plausible explanation may be found in the extremely small sample investigated by Logan and Kronfeld. Their original

sample was 25, with only 9 infants over one year of age, and half less than 3 months old at the time of death. Comparing Table I in Logan and Kronfeld with their ranges, many of the stages of tooth formation could not have been based on more than 2 specimens! Now, it is a cardinal principle that the extreme range is a function of sample size.[31] Such samples as were originally studied could hardly indicate the true extent of variability. Our samples are, in all probability, from 10 to 100 times as large as those originally responsible for the "ranges" for particular formation stages of individual teeth.

It is not our intention to slight the work of Logan and Kronfeld, which has not been surpassed. The original Logan-Kronfeld data were supplied to guide oral surgeons in the management of cleft-palate cases: these original investigators did not claim to provide "norms," in the usual sense. They stressed the *relative* nature of the values given, and the possible effects of growth, nutrition, etc. (Ref. 13, p. 380). Later authors have seized on the Logan-Kronfeld values rather uncritically, often without even adequate bibliographic mention, and without observing that histologically-determined values might not be applicable in routine pediatric examinations. What was originally advanced, quite tentatively, as new data properly contradicting the old tables of Pierce[7] and Black[8] became established by successive authors as authoritative normative values.

We would be open to similar criticism if we were to extoll our present values as "norms." Although the population sampled is a healthy one, and the sample size fair for some stages of some of the teeth, not all of the percentiles are based on 150 individuals or more. Our combined-sex values are, in some cases, derived from but 32-87 cases, and sex-specific values are, in some instances, necessarily based on as few as 14 examples. The sample, thus, falls short of the minimum 100 cases for each sex for each stage of each tooth that, ideally, would be needed. Nevertheless, the present sample is quite adequate to support the generalizations given above.

SUMMARY

1. Variability in the age of occurrence of 3 stages of tooth formation was investigated on serial oblique-jaw roentgenograms of 255 Ohio-born white children.

2. For the mandibular molar and premolar teeth, variability of tooth formation was almost perfectly correlated with the median age-for-stage ($r = 0.96$). Although later-forming teeth were more variable than earlier-forming teeth, relative or percentage variability was nearly constant throughout.

3. Boys and girls proved to be approximately equal in the variability of the 3 formation stages of the 5 mandibular teeth considered.

4. Comparing the 5th-95th percentile ranges from the present study to the "ranges" cited by most authorities, the ranges obtained here were 3 times as great as those ordinarily quoted.

5. Differences between the present findings and the "ranges" commonly given were attributed to the extremely small samples previously studied. In

many cases published "ranges" had been based on no more than two specimens of unstated sex and of questionable developmental normality at the time of death.

6. When the present data on tooth formation were compared with data on other developmental measures, relative variability proved to be of the same order of magnitude as tooth eruption, sexual maturation, etc., but somewhat less than that for osseous development.

7. These findings indicated the need for more adequate data on the variability of tooth formation, ideally based on samples of not less than 100 boys and girls for each developmental stage of each tooth, respectively.

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ANALYSIS OF AN ANOMALOUS OCULODENTOFACIAL PATTERN IN NEWBORN RATS PRODUCED BY MATERNAL HYPERVITAMINOSIS A

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INTRODUCTION

THE influence of prenatal factors upon the dentofacial complex has been studied in many animal experiments. Geneticists observed various deviations from the normal in inbred strains of mice[1] and adverse prenatal environmental influences also were shown effective in the production of dentofacial anomalies. We described previously a syndrome of skeletal and dental malformations attributable to maternal riboflavin deficiency.[2, 3] Recently, we encountered a different pattern of anomalies in the offspring of female rats fed excessive doses of vitamin A in early pregnancy. Cohlan,[4] in 1954, produced in rat fetuses gross developmental malformations of the head such as exencephaly, cleft palate, shortening of the mandible and maxilla, hydrocephaly and others by administration to their mothers of from 25,000 to 75,000 I.U. of vitamin A on the seventh to tenth day of gestation. Among the deformed young, specimens with large open eyes were observed, an anomaly not previously recorded. Giroud and Martinet[5] repeated and extended these experiments with similar results. In our laboratory the effectiveness of this teratogenic method was also confirmed.

Fig. 1 shows the external appearance of a normal newborn rat (*A*) which can be compared with a young produced by riboflavin deficiency (*B*), and one with hypervitaminosis A (*C*). Specimen *B* is characterized by marked shortening of the mandible, protruding tongue, and pointed snout. Specimen *C* shows a striking exophthalmia of an "open eye," shortening of both the mandible and maxilla resulting in protrusion of the tongue.

At first, it was thought that specimens with protruding eyes had essentially ocular anomalies, but histologic sections revealed that the eyes, although large and lidless, were almost normal in structure. However, there were skeletal anomalies of the face which we hold responsible for the protrusion of the eyeball. It is the purpose of this publication to describe the dentofacial malformations associated with these open and protruding eyes.

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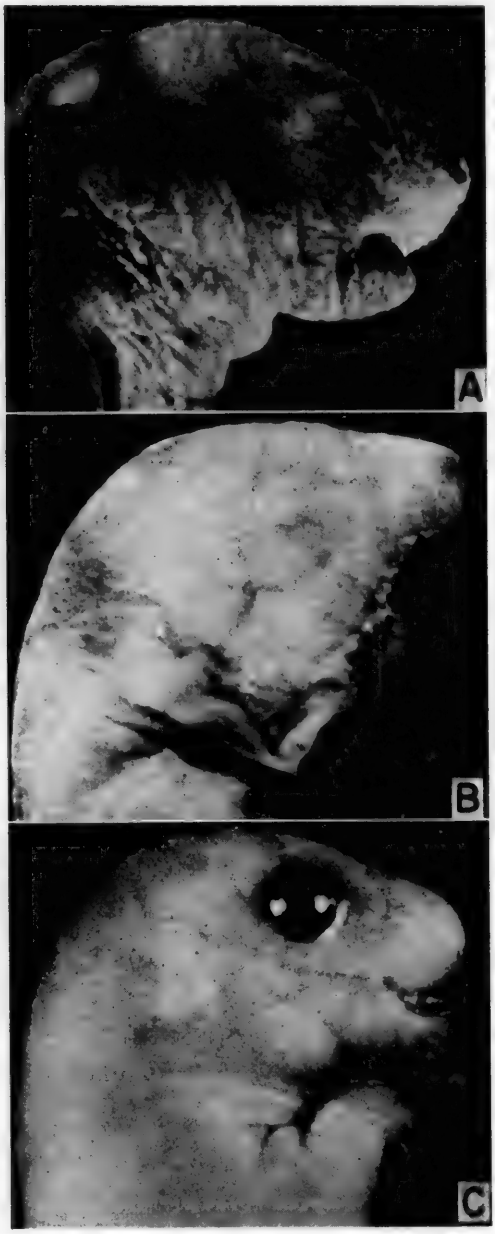

Fig. 1.—*A*, Head of normal newborn rat. *B*, Head of newborn rat, offspring of riboflavin-deficient mother, showing mandibular retrognathia with protruding tongue. *C*, Head of newborn rat, offspring of mother with hypervitaminosis A, showing open exopthalmic eye, shortening of upper and lower jaws, and protruding tongue.

PROCEDURE AND OBSERVATIONS

The specimens described and analyzed in this study were obtained by single doses of vitamin A (75,000 to 150,000 I.U.) administered orally to pregnant rats on the ninth or tenth or eleventh day, respectively. Eight specimens with large and protruding open eyes were serially sectioned. The dentofacial anomalies of the sectioned animals varied; they were analogous in some features but not in others. Three sectioned specimens were chosen as examples of the anomalies encountered in this syndrome.

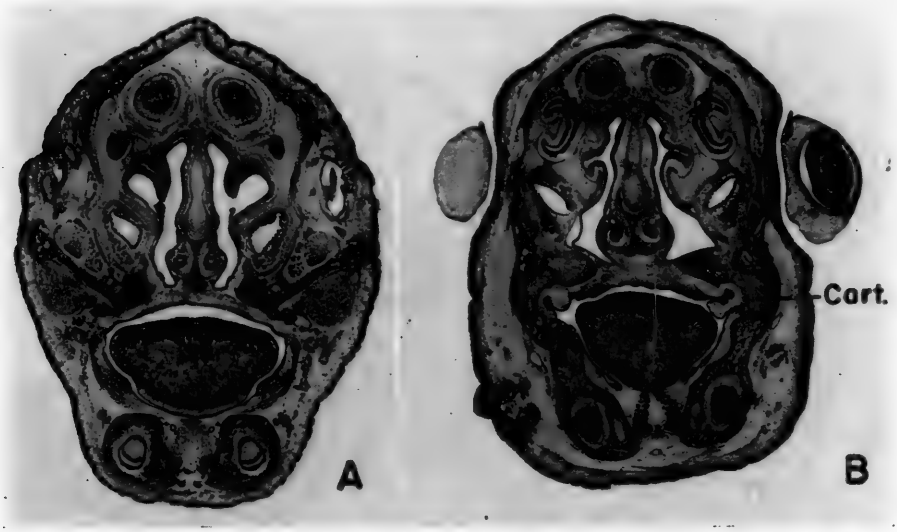

Fig. 2.—Frontal sections through region of the interdental diastema at the level of the anterior orbits. *A*, Normal animal. *B*, In abnormal young, produced by maternal hypervitaminosis A, eyes are exophthalmic and overhang the side of the head at this level. Observe: abnormal cartilage (*CART*) and the maxillomandibular ankylosis on the left side.

In Fig. 2, frontal sections of a normal and an abnormal newborn rat are compared. In this figure, sections through the region of the snout are reproduced which correspond to each other as much as possible. In the control (Fig. 2, *A*), the nasal cavity is large above and narrower below. The nasoturbinal is present and separated by a wide meatus from the maxilloturbinal which is attached to the lateral wall. The maxilla sends a process superolateral which ends in a swelling, the zygomatic process. This bony ledge forms the inferior wall of the orbit and supports the structures about the eyeball. No cartilage is present in or immediately inferior to the maxilla. Below the zygomatic process is a muscle mass, the masseter. The orbit is covered by the lids which are sealed at the palpebral fissure. Above the nasal cavity are the olfactory lobes of the brain roofed by the frontal bones. The floor of the nose is separated from the mouth by a membranous structure which is without bone. The section passes through the interdental diastema posterior to the upper incisors. The lower incisors which extend further posteriorly than the upper are seen.

An extreme case of exophthalmos associated with anomalies of the maxilla is represented by Fig. 2, *B*. In this abnormal animal, the lower part of the common nasal meatus is wider and the upper part narrower than in the control. The nasoturbinal and the maxilloturbinal approach each other, so that the intervening meatus is almost occluded. The maxilloturbinal is turned upward. The maxilla is represented by a medial part, the palatine process, and a lateral part, the maxillary body. These two parts are separated by a cartilage (Fig. 2, *B—Cart.*) which is absent in the control. The osseous process which extends superolateral in the control, forming the floor of the orbits, is absent in the experimental animal and the orbital contents are without support inferiorly.

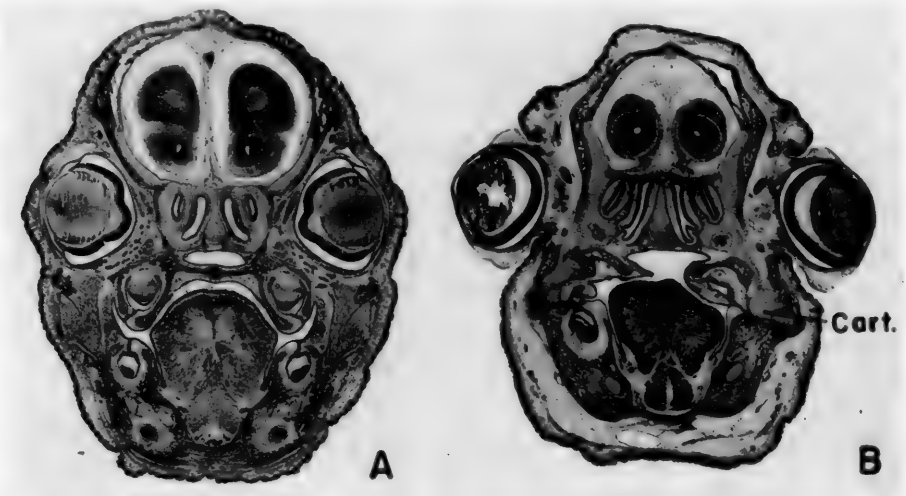

Fig. 3.—Sections through the region of the first molar teeth. *A*, Normal animal. *B*, Abnormal animal. Note: the presence of only one molar tooth, abnormal cartilage (*CART*), cleft palate, canalized polyp under the left palatine shelf, and the protruding and lidless eyes.

The rudimentary lateral part of the maxilla is connected by a narrow bony bridge with the mandible, resulting in ankylosis. This section, like that in the control, passes through the interdental diastema. In the left lateral recess of the oral cavity, a pedunculated polyp can be seen. The parts of the eyes seen in this section seem to be separated from the head. This effect is due to the forward bulging of the globe which is not connected at this level with the head proper.

A posterior section of the same specimen is illustrated in Fig. 3, *B* which, in contrast to the control (Fig. 3, *A*), shows the palatine processes unfused, resulting in a posterior cleft palate. A mass of heterotopic maxillary cartilage located between the maxilla and mandible is well developed. In contrast to the control, the alveolar processes and the first molar teeth are missing in the maxilla. The first molar is also absent in the left mandible, but, in the right mandible, a molar tooth (the only molar of this specimen) can be seen. An

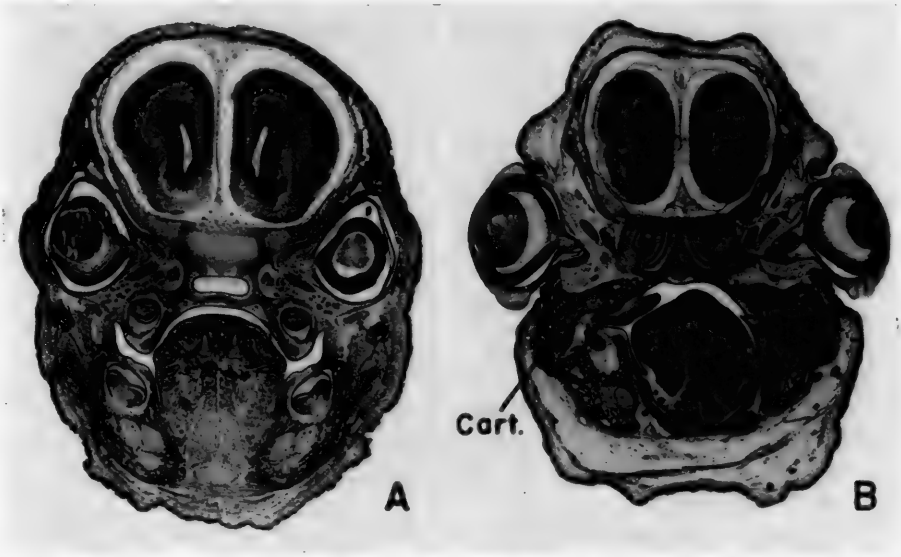

Fig. 4.—Sections through the level of the optic nerve. *A*, Control animal. Note orbital floor and masseter muscle. *B*, Abnormal animal. Note: defective orbital floor, abnormal cartilage (*CART*), cleft palate, and posterior end of molar tooth and lamina in the right mandible.

Fig. 5.

Fig. 6.

Fig. 5.—Abnormal animal produced as a result of maternal hypervitaminosis A. Section through region of first molar teeth. Note: absence of upper right first molar tooth, abnormal cartilage, and cleft palate.

Fig. 6.—In this abnormal animal, the ocular anomaly is unilateral and on the right side. Observe: the heterotopic cartilages on both sides, the presence of all four molars with the upper ones reduced in size.

opening can be noticed in the mucosal polyp below the left palatine process. The lidless eyes show marked protrusion and overhang the cheeks. Sections of the ocular muscles can be seen.

The section shown in Fig. 4, *B* goes through the optic nerve on the right side. Except for protrusion of the globes and absence of the lids, the eyes are normal. The orbits are supported inferiorly by the heterotopic cartilage. The posterior extremity of the right mandibular molar is sectioned but there are no traces of the other molars in this or in subsequent sections. The nasal cavity is still visible in this section which goes through the right optic nerve while, in a corresponding control (Fig. 4, *A*), the nasopharyngeal duct is already seen. This discrepancy is due to the shortening of the abnormal head.

Another specimen which has protruding eyes, not seen in the section reproduced, shows a heterotopic cartilaginous bar and a somewhat different dental pattern (Fig. 5). On the left side there is a small upper first molar tooth, but there are no molars on the right. In subsequent sections, no second and third molars could be detected on the left. In Fig. 5, a posterior cleft of the palate is also present.

A specimen with unilateral open eye is of interest because it permits comparison of the two sides in one section. Fig. 6 shows the right eye larger than the left, and rudimentary lids over the right eye. The left eye appears normal. Heterotopic cartilaginous bars are present on both sides but they are smaller than those described in previous specimens. Four molars are present but the upper ones are somewhat hypoplastic. This animal represents a transitional stage from the marked anomaly, described before, to the normal condition.

DISCUSSION

The experimental disturbance of the abnormal specimens described in the preceding pages resulted in an interesting pattern of oculodentofacial anomalies. The outstanding features of the abnormal pattern produced by hypervitaminosis A were exophthalmus, maxillomandibular ankylosis, the presence of heterotopic cartilage in the maxilla, and the absence of some molar teeth. The syndrome manifested itself chiefly in derivatives of the mandibular arch and it appears that the eye was secondarily involved because of its regional relationship to this area. Mesodermal and ectodermal structures were affected and it is likely that the dental anomalies are attributable to the anomalies of the underlying mesodermal tissues. The pattern produced by hypervitaminosis A differed from that of riboflavin deficiency in several respects. In the young produced by riboflavin deficiency, only the incisor teeth were affected, the lower more than the upper. The distal portions of the jaws were particularly reduced in length. In the young, as a result of hypervitaminosis A, more proximal portions of the jaws were involved and their reduction resulted in anomalies of molar teeth. It is of interest that different syndromes of multiple congenital anomalies can be produced by different prenatal environmental disturbances. The syndrome described above, and produced by prenatal hypervitaminosis A, results in absence of some of the molar teeth, an anomaly not

described in the riboflavin-deficiency syndrome. A deficiency of molar teeth has been observed before in some strains of inbred mice. Grüneberg,[1] for instance, described absence of third molars in an inbred strain (CBA). Although these molar anomalies were due to a genetic disturbance, the manifestation of the character seemed to be strongly influenced by intra-uterine conditions, since abnormal animals tended to occur grouped in certain litters.

The dependence of the development of the molar teeth upon genetic as well as environmental factors, as demonstrated in such animal experiments, illustrates the difficulties of simple interpretations of similar anomalies in man. Anomalies which occur spontaneously under certain genetic constellations can be imitated by environmental procedures and disturbances of the prenatal environment. It becomes increasingly evident that congenital defects are in many instances attributable to a combination of genetic and prenatal environmental factors.

SUMMARY

Oculodentofacial anomalies observed in the young of rats with hypervitaminosis A were analyzed. It was found that exophthalmic eyes were essentially normal in structure but protruded as a result of congenital malformation of the orbital floor. Anomalies of the maxilla and the molar teeth were also seen. These anomalies were different from those produced by maternal riboflavin deficiency previously described.

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EFFECT OF REPEATED ADDITIONS OF SUBOPTIMUM AMOUNTS OF A REQUIRED VITAMIN ON GROWTH AND ACID PRODUCTION OF LACTOBACILLI

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A COMMONLY held concept of the carious process is that it is initiated primarily by acid resulting from sugar fermentation caused by certain oral microorganisms at definite sites, as in plaques on the tooth surface. The acid production may conceivably be continuous but is often thought of as more or less intermittent, depending upon the availability of fermentable sugar in the environment as well as the presence of other nutrients required by the microorganisms. Lactobacilli and streptococci are the bacteria usually believed to be chiefly responsible for acid production.

It is known from studies of nutritional needs of microorganisms that growth can be prevented by withholding an essential nutrient, such as a required vitamin or amino acid, which the microorganism itself is unable to synthesize. In most of the work dealing with growth response of microorganisms to a required vitamin, the full amount of the vitamin to be used in the experiment has been added to the medium not later than the time of inoculation. Thus, the vitamin is available at once for active cell metabolism and growth, resulting in maximal growth and acid production, or a lower level if a suboptimum amount of vitamin has been supplied. In the latter case, growth and acid production cease at a definite point depending on the amount of required nutrient available.

The question may be raised as to what happens if an acid producing oral microorganism receives several intermittent small amounts of a required nutritive substance, rather than getting a larger supply all at one time. In the present study, an attempt has been made to answer this question by taking advantage of the known vitamin needs of lactobacilli.[1-3] Repeated or multiple additions of a small amount of vitamin, or any other essential nutrient, might simulate more nearly the conditions under which lactobacilli must develop, at least at times, in certain sites in the mouth.

METHODS

Basal Medium.—The basal medium consisted of the following substances per 1,000 ml. of medium: 5 grams acid hydrolyzed purified casein, 10 grams glucose, 6 grams anhydrous sodium acetate, 50 mg. L-tryptophane, 100 mg. each

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of DL-serine and DL-threonine, 50 mg. cysteine.HCl, 10 mg. each of adenine sulfate, guanine hydrochloride, thymine, and uracil, 3 grams K_2HPO_4, 200 mg. $MgSO_4 \cdot 7H_2O$, 10 mg. $FeSO_4.7H_2O$, 7.5 mg. $MnCl_2.4H_2O$, and vitamins. Sodium was also supplied in the form of sodium hydroxide to bring the medium to the desired pH. This resulted in the addition usually of about 0.23 gram of sodium per liter, along with the sodium supplied as sodium acetate. The vitamins normally used per 1,000 ml. of medium were: 10 μg biotin, 1,000 μg nicotinic acid, 200 μg pteroylglutamic acid, 100 μg para-aminobenzoic acid, 1,000 μg calcium pantothenate, 1,000 μg pyridoxal hydrochloride, 200 μg riboflavin, and 100 μg thiamine hydrochloride. Of the above substances, cysteine and thiamine were added from filter-sterilized solutions to individual tubes or flasks of medium at the time of inoculation, the thiamine being used only in the case of *Lactobacillus fermenti*, a heterofermentative type which requires thiamine. Another addition to the medium, when *L. fermenti* was employed in the experiments, was a mixture of 3 pentoses: L-arabinose, D-xylose, and D-ribose. These were autoclaved together in distilled water solution and added to the tubes or flasks of medium in an amount to give a final concentration in the medium of 0.5 per cent of each pentose. The pentoses were added because of their stimulating effect on growth of *L. fermenti*, which otherwise may be rather slow at times.

The tests with each of the several vitamins in repeated, suboptimal quantities made necessary some departures from the foregoing amounts in the case of the particular vitamin used in each experiment. For example, when the response to repeated small amounts of biotin was determined, the amount of biotin was adjusted as specified in later experiments, but the other substances were all supplied in the amounts listed above.

The medium was adjusted to pH 6.8 with sodium hydroxide. It was dispensed usually in 125 ml. amounts in 250 ml. flasks. After sterilization in the autoclave the pH was about 6.4 to 6.5.

Cultures.—Strains of oral lactobacilli of both the hetero- and homofermentative types, and several American Type Culture Collection strains were used. All cultures were kept in stock in tomato juice–yeast extract (TJY) medium of the composition described by Orland.[4] For inoculation of tests, a 24-hour TJY culture, made from a recent transfer of the stock culture, was used; 1 ml. of a 1:100 saline dilution was introduced into 125 ml. of medium in a flask.

Procedure.—Each experiment consisted of 2 or 3 flasks of basal medium from which the particular vitamin under investigation had been omitted. One flask, just before inoculation, received an amount of the vitamin sufficient to support good growth of the lactobacillus. No more vitamin was added to this culture. It served as the control which received its vitamin supply in the usual way, i.e., all of it at the start. The other one or two flasks, termed experimental, received a distinctly suboptimal amount of the same vitamin, enough for only fair or moderate growth. All flasks were incubated at 36° C. After 3 days, samples were withdrawn from the flasks for (a) Lumetron readings of turbidity, (b) plate counts of viable cells using either TJY or the APT medium of Evans and Niven,[5] and (c) pH determinations. The TJY agar

was used in most cases, but APT agar medium was found preferable for plating some strains of heterofermentative lactobacilli which formed only small colonies on TJY plates.

After withdrawal of the sample for the above determinations, another small amount of the vitamin was added to the experimental flasks, with or without neutralization of acid as described below. Nothing was added to the control flask nor were any adjustments made in it. The procedure was repeated, usually 4 times, at intervals of several days until the experimental flasks received the same total amount of the vitamin that was placed in the control flask at the start.

In some cases the acid formed in the experimental flask was neutralized to pH 6.8 with sterile 1 N sodium hydroxide solution just before the next succeeding addition of vitamin. Thus, the lactobacillus was afforded an opportunity to carry on metabolism at approximately the original pH of the medium, if able to do so. It should be pointed out that, although the acid was neutralized, the cells were continually exposed to other metabolic products which had accumulated as a result of earlier growth. In other experiments the acid produced by the lactobacillus, after each supply of vitamins, was not neutralized. In these cases, the cells, on each succeeding addition of the small amount of vitamin, must carry on metabolism, if possible, in an environment becoming gradually more acid and which contained other products of growth as well.

RESULTS

Fig. 1 shows the response of *L. casei* 7469 to 5 separate additions of suboptimal amounts of biotin given at 3-day intervals starting at the time of inoculation. Turbidity resulting from growth of the lactobacillus is shown by the per cent light transmission, in which decreasing values represent greater turbidity. It is evident that in the control *L. casei* grew readily in the presence of 0.0001 μg biotin per milliliter. At the third day after inoculation, it had attained a turbidity of about 33, a pH of 4.1, and there were 455 million cells per milliliter. The amount of biotin used is not quite sufficient for maximal growth. It may be added parenthetically that by increasing the amount of biotin it is possible to reach somewhat higher cell counts and a pH of 3.8 or thereabouts in this medium. The object of the work, however, was to compare results with the same amount of vitamin added in different ways. Thus, the quantity of vitamin used in the controls was determined by the total amount planned for use in the multiple addition experiments, not by the amount needed for maximal growth and acid production.

When the vitamin is added in small increments, response of the lactobacillus to the initial amount is distinctly limited; the turbidity, acidity, and cell counts are all lower. In the flask in which the acidity was not neutralized, there is a gradual, though not marked, increase in turbidity over the remaining period as additional small amounts of vitamin are added. There is also an increase in acidity from pH 4.9 at 3 days to 4.1 at 15 days. In this case, the eventual pH value reached the same level as the control where all the vitamin was added initially, although in many other similar experiments this was not the

case. Evidently the small increments of vitamin serve to support some continuing cell metabolism and additional acid production over this period, in spite of the fact that the cells are in a distinctly acid environment. The highest count of viable cells under these conditions was recorded at 5 days, or after the second addition of vitamin, with a decrease in numbers by the ninth day; however, the numbers are distinctly greater than in the control flask at 5, 9, and 12 days. In the control flask which grows out at once and attains pH 4.1, the cells die rapidly at 36° C. after peak growth is attained.

In the remaining experimental flask of Fig. 1, the acidity was readjusted to pH 6.8 at each 3-day interval. Thus, the pH of 4.5 at 6 days and pH 5.1 at 9 days represent more acid production by the cells after the 3-day and 6-day neutralization and addition of more vitamin. During this interval, the turbidity increased but slightly and the count of viable cells decreased after the third day.

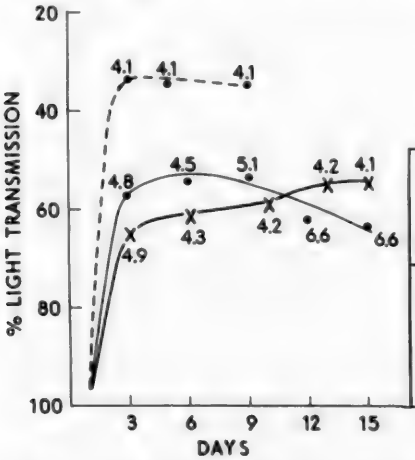


	PLATE COUNTS millions/ml.		
	Control	Multiple Additions	
Days		Not Neut.	Neut.
0	.04	.04	.04
3	455.	178.	155.
5	2.7	407.	84.
9	.6	103.	3.
12	< .01	1.	5.
15	< .01	.02	.2

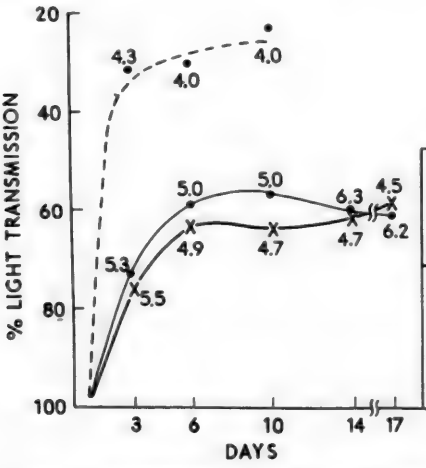
Fig. 1.—Biotin and *L. casei* 7469. Biotin, 0.00002 μg per ml., added at 0, 3, 6, 9, and 12 days to total of 0.0001 μg per ml. Control received 0.0001 μg per ml. at 0 time. pH values of the cultures at different intervals are shown on the turbidity curves, i.e., 4.1, 4.8, 4.9, etc. Plate counts for viable cells are given at right. —x——x— = multiple additions of vitamin, acid not neutralized. —●——●— = multiple additions of vitamin, acid neutralized. --●---●-- = control.

However, some metabolic activity evidently occurred and more acid was formed during this interval. After 9 days, however, little or no acid was produced after neutralization and addition of more vitamin. The viable cell count is low at this time, although it will be noted that toward the end of the experiment the count is higher than in the other experimental flask which was not neutralized at each interval. Evidently the periodic neutralization of acid, together with addition of a little more vitamin, served to prevent as rapid a decrease in numbers as in the other experimental flask or in the control. The same effect often was seen in other experiments with different strains of lactobacilli and different vitamins.

A number of additional experiments similar to the one shown in Fig. 1 were also performed with biotin and other lactobacilli: *L. casei* strains 4 and 46, *L. fermenti* strains 6 and 41. These results are not given in detail but,

in general, were similar to those of *L. casei* 7469 in Fig. 1. In the instances where cultures received multiple additions of small amounts of vitamin, the turbidity and the acidity were always somewhat less and, in some cases, distinctly less than in the controls which received all of the vitamin at the start. However, the number of viable cells at the later time intervals was greater where multiple additions of vitamin were received than in the controls.

Fig. 2 summarizes the results obtained in a similar experiment with nicotinic acid and *L. casei* 4, a lactose-negative oral strain. Following multiple additions of nicotinic acid neither the turbidity nor the pH values of the experimental cultures ever equaled those of the control which received all of the nicotinic acid at the start. The unneutralized experimental flask eventually reached pH 4.5 after periodic vitamin additions. In the lot that was periodically neutralized the cells succeeded in bringing the pH to 5.0 on two occasions but later produced only slight changes in pH. But, here again, the number of viable cells in both of the multiple addition cultures was greater than in the control from the sixth day to the end of the experiment.



Day	PLATE COUNTS millions/ml. Control	Multiple Additions Not Neut.	Multiple Additions Neut.
0	.03	.03	.03
3	360.	128.	125.
6	52.	95.	135.
10	1.4	20.	29.
14	.5	2.	17.
17	.07	0.8	1.

Fig. 2.—Nicotinic acid and *L. casei* 4 (lactose negative). Nicotinic acid, 0.005 μg per ml., added at 0, 3, 6, 10, and 14 days for total of 0.025 μg per ml. Control received 0.025 μg per ml. at 0 time. pH values and plate counts are shown as in Fig. 1. —x——x— = multiple additions of vitamin, acid not neutralized. —●——●— = multiple additions of vitamin, acid neutralized. --●--●-- = control.

Similar experiments with repeated additions of suboptimal amounts of nicotinic acid and other lactobacilli were also included in the work. Since the results in general were similar to those given in Fig. 2 they are not shown in detail. The lactobacilli used in these other tests were *L. casei* strains 45, 96, and 7469, and a lactose-negative strain, 1009.

Figs. 3 through 5 show results of similar experiments with riboflavin, calcium pantothenate, and thiamine. In each case, multiple additions of the vitamin were made until the total amount equaled that of the control tube. The experimental medium was neutralized just before each periodic vitamin addition; unneutralized lots were not included in these experiments. Aside from

certain differences in detail, the general picture of the results is much the same, irrespective of the vitamin and the lactobacillus used. The amount of growth as shown by turbidity is distinctly less when the vitamin is added in several small amounts. In some instances, the pH of the experimental lots approaches, but does not equal, that of the control during the earlier part of the experiment.

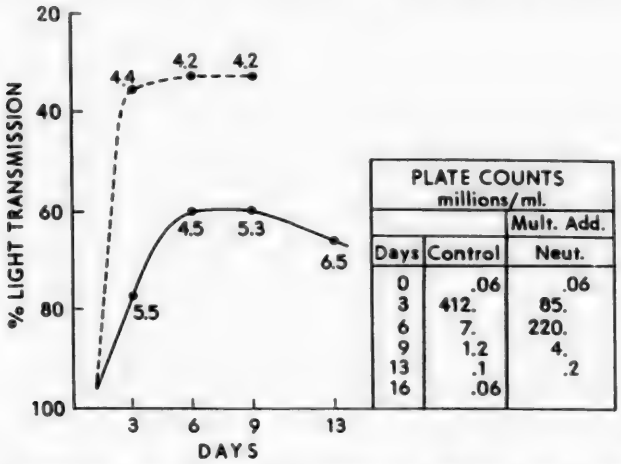


PLATE COUNTS millions/ml.		
		Mult. Add.
Days	Control	Neut.
0	.06	.06
3	412.	85.
6	7.	220.
9	1.2	4.
13	.1	.2
16	.06	

Fig. 3.—Riboflavin and *L. casei* 46. Riboflavin, 0.005 μg per ml., added at 0, 3, 6, 9, and 13 days for total of 0.025 μg per ml. Control received 0.025 μg per ml. at 0 time. pH values and plate counts shown as in Fig. 1. —●——●— = multiple additions of vitamin, acid neutralized. --●---●-- = control.

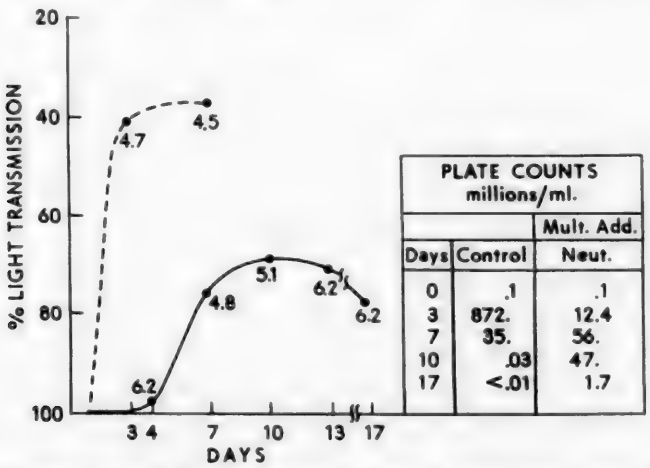


PLATE COUNTS millions/ml.		
		Mult. Add.
Days	Control	Neut.
0	.1	.1
3	872.	12.4
7	35.	56.
10	.03	47.
17	<.01	1.7

Fig. 4.—Pantothenate and *L. fermenti* 6. Calcium pantothenate, 0.004 μg per ml., added at 0, 4, 7, 10, and 13 days for total of 0.02 μg per ml. Control received 0.02 μg per ml. at 0 time. pH values and plate counts shown as in Fig. 1. —●——●— = multiple additions of vitamin, acid neutralized. --●---●-- = control.

This is seen in the case of riboflavin and *L. casei* 46 where the pH was 4.5 at 6 days compared with 4.2 in the control, also in the experiment with pantothenate where pH 4.8 was reached in comparison with 4.5 of the control. *L.*

fermenti strains 6 and 41 are heterofermentative lactobacilli and the pH values reached by these organisms are not as low as those of homofermentative lactobacilli. *L. fermenti* 6 often grew somewhat more slowly in the medium used for the tests than many other lactobacilli. In many of the above cases, the

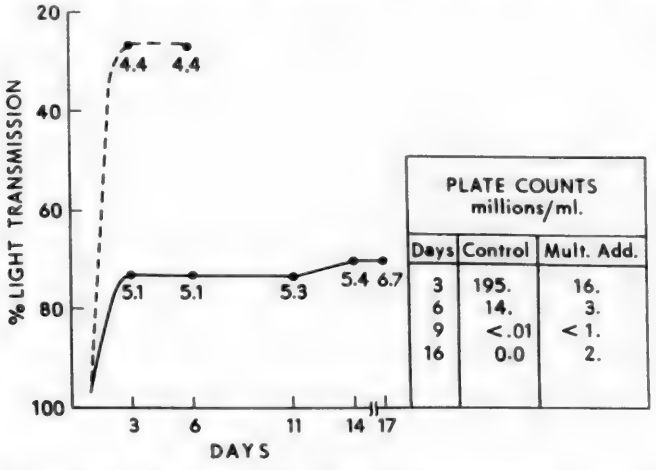


Fig. 5.—Thiamine and *L. fermenti* 41. Thiamine hydrochloride, 0.002 μg per ml., added at 0, 3, 6, 11, and 14 days for a total of 0.01 μg per ml. Control received 0.01 μg per ml. at 0 time. pH values and plate counts shown as in Fig. 1. —●——●— = multiple additions of vitamin, acid neutralized. --●---●-- = control.

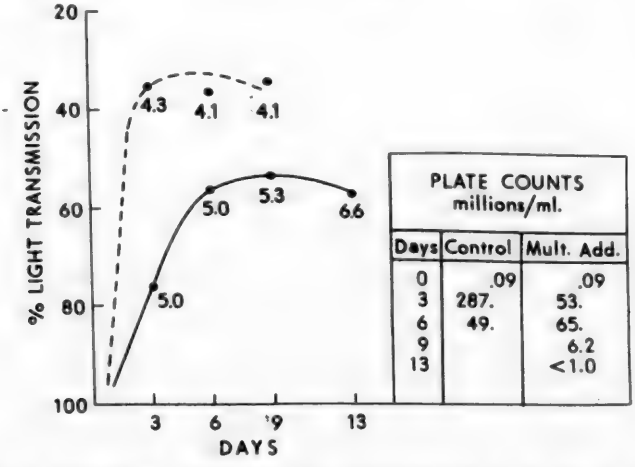


Fig. 6.—Tryptophane and *L. casei* 45. L-tryptophane, 0.2 μg per ml., added at 0, 3, 6, 9, and 13 days for total of 1.0 μg per ml. Control received 1.0 μg per ml. at 0 time. pH values and plate counts shown as in Fig. 1. —●——●— = multiple additions of tryptophane, acid neutralized. --●---●-- = control.

lactobacillus, after readjustment of the medium to pH 6.8 and the addition of another small amount of vitamin, did not succeed in lowering the pH as much as in the earlier periods of growth. Toward the end of the experiments there is evidently little metabolic response to new additions of vitamin.

One amino acid was used. L-tryptophane is required for growth of most lactobacilli and consequently can be used for the same type of experiment. In this case all required vitamins were supplied in adequate amounts but the supply of tryptophane was regulated. In the experiment shown in Fig. 6, the 1.0 μg per milliliter of the control supports good growth, although not quite optimal, of *L. casei* 45. Two-tenths microgram per milliliter of L-tryptophane supports distinctly suboptimal growth. Later additions of this same amount at 3-day intervals did not support growth or pH levels equal to those of the control and the over-all picture of results for tryptophane is the same as for the individual vitamins.

SUMMARY

Growth response and acid production by lactobacilli to multiple suboptimum amounts of a required vitamin, supplied over a period of 12 days or more, were compared with the response when the same total amount of vitamin was supplied at the time of inoculation in the conventional way. A basal medium of acid hydrolyzed casein, glucose, acetate, salts, and supplements was used.

The periodic additions of small amounts of a required vitamin did not support as full growth as the same total amount of vitamin given at the start. However, the periodic additions evidently caused some metabolic stimulus, and more viable cells usually were found toward the end of the experimental period under such conditions.

In lactobacillus cultures supplied the periodic small amounts of vitamin, pH values usually were not as low as, although at times they approached, the pH values reached when all the vitamin was supplied at the start. When the acid resulting from cell metabolism supported by repeated small amounts of vitamin was neutralized periodically, lactobacilli were able to again lower the pH over the first few periods as more vitamin was supplied, but in later periods little change in pH resulted when additional small amounts of vitamin were supplied.

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STREPTOCOCCI IN DENTAL CARIES

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THE specific bacterial etiology of dental caries is still uncertain, in spite of the enormous amount of work directed to its elucidation. The only undisputed evidence available to date is that caries cannot occur in the absence of microbes.[1]

The problem of bacteria associated with caries has already been investigated on three successive levels, comprising the direct environment of the carious lesion: (a) exploration of the vast flora of the saliva, (b) identification of the bacteria found in the dental plaque, and (c) study of bacteria harboring within the lesion itself.

Early investigators, such as Kligler[2] and others,[3] realized the bewildering character of the oral flora, but regarded the lactobacillus as probably a particularly significant member of the carious flora. Hence, they focused their attention on the lactobacilli, usually abundant in the saliva before the appearance of clinical caries.

Many others were inclined to believe that streptococci or Actinomyces play a role in caries initiation. Still, there is at present little direct evidence on which a decision could be made as to the respective importance of the organisms mentioned—and sporadic accounts of new screenings of oral bacteria[4, 5] on a broader scale are stimulating and promising.

A fourth level has comparatively recently focused the attention of investigators.[6] It is mainly the search for bacteria having the ability to invade apparently intact tooth structure in vivo, and presumably involved in initial caries, or leading its progress. MacGregor and associates[11] mention several investigators as stating that bacteria penetrate far ahead of actual decalcification and decay.

Several distinct types of bacterial invasion of tooth structure have been repeatedly observed in *histologic sections* by optical and electron microscopy.[7-9]

Our investigation was carried out in two directions, with the aims in view: first, to inquire into bacterial invasion by acceptable *bacteriologic* criteria; and, second, to isolate the primary invaders, and to see whether they belong to a well-defined group.

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MATERIALS AND METHODS

Experimental.—In order to narrow down the wide spectrum of the salivary or plaque bacteria and avoid contamination by secondary bacteria, we have worked out a direct method of obtaining enamel or dentin scrapings from the superficial lesion or from the bottom layer of small cavities. Deeper cavities with extensive decay were generally avoided, for fear of contamination.

Method of Collecting Material.—Teeth to be restored were isolated, cavities excavated in a routine manner of cavity preparation. The floor of the cavity was washed with alcohol, a fresh sterile bur was mounted, and scrapings from the sound adjacent structure were let to fall down on a sterile dental mirror, from which they were then dropped into sterile broth.

We could apply this technic conveniently with upper teeth only, and consequently no mandibular teeth were used in this investigation. In some cases, we took material from the pigmented, although apparently sound, enamel.

Media.—During the first stage of this work, the scrapings were cultivated in Difco brain heart infusion broth enriched with 5 per cent Seitz-filtered tomato juice, and supplemented with bromcresol green as pH indicator (BHT).

The final reaction of the medium is around pH 6, with the indicator tinting it green. The main advantage of this medium for the present purpose lies in its low selectivity and the wide range of redox potentials it offers. Practically all the organisms usually encountered in the oral microflora can grow almost equally well in this medium. It seems reasonable to suppose that a medium which enables growth of the anaerobic clostridia as well as that of aerobic bacilli, fastidious streptococci, as well as various lactobacilli and diphtheroids, will offer suitable conditions for any other type of oral bacteria the redox and nutritional requirements of which lie between those of the above organisms.

The results obtained with the medium described above are given in Column 2 of Table I.

TABLE I

ORGANISMS ISOLATED FROM DENTINAL SCRAPINGS IN 3 SEPARATE SERIES OF CLINICAL CASES

ORGANISMS ISOLATED	NO. OF CASES			
	1ST SERIES	2ND SERIES	3RD SERIES	TOTAL
Pure streptococci	25	9	9	43
Streptococci + lactobacilli		2		2
Streptococci + others		1	7	8
Pure lactobacilli			1	1
Diphtheroids			1	1
Micrococci	5			5
None (material found sterile)	5	2	5	12
Totals	35	14	23	72

Second Series.—After it became clear that streptococci could be fairly regularly isolated by this method, it was considered advisable to employ more stringent cultural conditions to rule out several possible sources of error.

A few drops of the original brain heart (BH) broth inoculated with the dentinal scrapings were transferred (1) to a Difco thioglycollate broth tube, in order to make absolutely sure that no anaerobes would be missed, and (2) to 2 Petri dishes containing Difco brain heart infusion agar, supplemented with 0.5 per cent yeast extract, in order that no organisms would be overlooked as a result of overgrowth in the liquid media.

Column 3 of Table I shows the results obtained in a series of 14 cases in which the modified cultural methods were used. Streptococci were found in every one of the 12 positive cultures: in 3 cases—together with lactobacilli and other bacteria, in the other 9—in pure culture. The BH plates, on which the original material was also seeded, remained sterile in these 9 cases. It was, therefore, concluded that no other aerobic organisms were present in the BH tubes besides streptococci (streptococci failed to grow on the solid medium—see below).

In the original tube, the streptococci were apparently found in small numbers, since growth was obtained sometimes in the original tube, while no growth was obtained when this material was diluted 1:10 to 1:50.

CHARACTERISTICS OF THE STREPTOCOCCI ISOLATED FROM THE HARD BASE OF CARIOUS LESIONS (MORPHOLOGICAL, CULTURAL, AND BIOCHEMICAL)

The streptococci appeared as microaerophilic gram-positive spheres of about 0.7 to 1.0 μ in diameter, in short or long chains, sometimes in pairs. They were pleomorphic, often appearing in short, ovoid rods with rounded ends. Growth in the deeper part of the BHT broth is diffuse, abundant, resembling bread crumbs with an optimum temperature of 36° to 38° C. They will not grow on primary isolation on BH plates, when in small numbers, but will grow well in thioglycollate broth, bringing down the reaction to about pH 5.0. On solid media the characteristic growth is scant, dry, and rough; growth on BH slopes is improved by addition of broth (BH) to prevent dryness. They will not disperse well in saline or broth.

In thioglycollate broth, these streptococci grew sometimes in a peculiar stalactite fashion (Fig. 1).

Of the 49 cases studied so far, 7 proved sterile, after prolonged incubation in all the media mentioned. It confirmed previous observations that scrapings of dentin obtained beyond the immediate base of the cavity are sterile. (This observation is of clinical importance in that an overzealous attempt to search these organisms down into sound dentin appears superfluous.)

It was of interest to determine whether these streptococci belonged to a homogenous group. Since the number of strains available from the previous work was not sufficient, we investigated 23 more clinical cases, in order to increase the number of strains in our collection. The media used in this third series were identical with those of the second series. The results obtained are given in Column 4 of Table I.

Twenty-three of the streptococcus strains isolated from the second and third series were sent for typing to Dr. R. E. O. Williams, Streptococcal Reference Laboratory, London. Eight of these strains failed to grow by the time

they reached London (among them 3 which had been lyophilized before dispatch), and could not, therefore, be tested biochemically. Fourteen of the 23 strains (including 3 of the strains which failed to grow in London) were

Fig. 1.—Stalactite growth of pure streptococcal culture in thioglycollate broth. Note growth starting a few millimeters below the surface, showing the microaerophilic character of the microorganism.

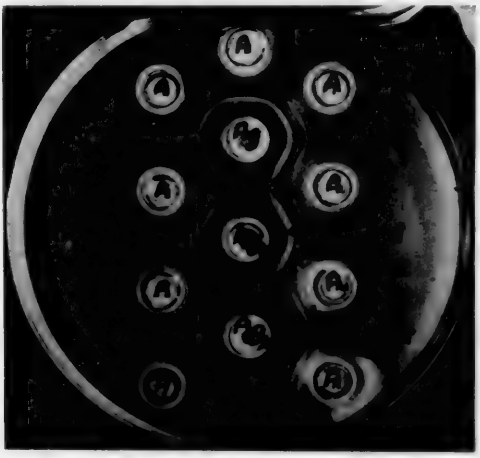

Fig. 2.—Agar diffusion pattern given by streptococcus strains against standard viridans antiserum. *A*, *Streptococcus viridans* No. 10/2 antiserum. *Ag*. *Streptococcus viridans* 10/2 antigen. Ag_1, Ag_2 = 2 different streptococcus antigens. 1—4, 4 precipitation lines.

tested by us serologically by the agar diffusion method of Ouchterlony. This was done as follows: a potent antiserum was prepared against one of the viridans streptococci (strain 10/2) by injecting rabbits with acetone-killed bacteria incorporated in Freund's adjuvants. The technics used in preparing the antigen for immunization, bleeding the rabbits, and setting up the agar diffusion tests have been described.[10]

The results of Dr. Williams' typing are summarized, together with the results of our serological tests in Table II. Because of the small number of strains used in this study, these results can only be regarded as of a very preliminary nature. They show that many of the streptococci isolated from the advancing front of the caries lesion belonged to the viridans group, and that most of these bacteria were either identical or related serologically. Strains were classified "identical" when giving the same number of four diffusion lines (Fig. 2) as the organism used in preparing the antiserum; and "related," when at least one line was obtained in agar against the standard viridans antiserum.

TABLE II

BIOCHEMICAL AND SEROLOGICAL TYPING OF STREPTOCOCCAL STRAINS

BIOCHEMICAL TYPING	NUMBER OF STRAINS				
	TESTED BIOCHEMICALLY (DR. R. E. O. WILLIAMS)	TESTED SEROLOGICALLY	IDENTICAL WITH STRAIN 10/2	RELATED TO 10/2	UNRELATED TO 10/2
*	*	3	–	–	3
No hemolysis	2	2	–	1	1
Unidentified viridans strains	7	7	4	2	1
Str. sanguis	3	2			2
Str. faecalis	3	–			–
Totals	23	14	4	3	7

*These were the 8 strains which failed to grow by the time they reached Dr. Williams' laboratory.

Competitive Effect of Streptococci and Lactobacilli in Mixed Cultures.— The repeated isolation of streptococci in pure cultures in liquid media raised the question as to whether this was due to overgrowth of other organisms present, in particular lactobacilli by streptococci.

In order to test this point, the following experiment was performed: 100-fold dilutions were prepared from 24-hour streptococci and lactobacilli cultures in thioglycollate broth. Two-milliliter quantities of each streptococcus dilution were added to tubes containing 2 ml. of the various lactobacilli dilutions, according to the scheme shown below. After 48 hours of incubation, gram-stained preparations were made from each tube, to ascertain the type of growth obtained. It was found that when large numbers of bacteria were mixed, both organisms grew in the tubes. With small numbers of bacteria, an approximately 100-fold difference was sufficient for the organism added in excess to completely overgrow the other (Table III).

This experiment was repeated 3 times, each time with a different pair of streptococcus-lactobacillus (both recently isolated from clinical cases of caries), with practically identical results.

Since the number of streptococci found in the advanced layer we were testing, subjacent the carious lesion, was very small, there can be no question of their being in large excess over lactobacilli. It was, therefore, concluded that our pure cultures were due to the presence of a single organism in the test culture, the streptococcus.

TABLE III

GROWTH OF STREPTOCOCCI AND LACTOBACILLI IN MIXED CULTURES

LACTOBACILLI DILUTIONS	STREPTOCOCCI DILUTIONS				
	UNDILUTED	10^{-2}	10^{-4}	10^{-6}	10^{-8}
Undiluted	M	M	L	L	L
10^{-2}	M	M	L	L	L
10^{-4}	M	M	M	L	L
10^{-6}	M	M	M	M	L
10^{-8}	S	S	S	S	O

Explanation: L = tubes showing lactobacilli in pure culture. S = tubes showing streptococci in pure culture. O = no growth. M = mixed cultures.

DISCUSSION

Experiments devised to demonstrate bacterial invasion of teeth have been reported before.[7, 8] The work of Harrison[6] is particularly relevant. To quote:

> As others have done before, we began to seek ways of finding a point at which the flora might be simpler, a point at which secondary invaders might be less likely to have come into the picture. By this method the material cultured was from the extreme forward margin and contained only such organisms as had sufficient invasive power to penetrate into the dentin, and theoretically might be assumed to have caused the lesion.

Most of the previous evidence by methods of histology and histopathology was static, as based solely on histologic examination of fixed specimens, and could, therefore, be argued against. The advantage of our method lies in the fact that there is no room for mistake as to the presence or identity of the organisms, for they can be cultivated and studied by conventional bacteriologic technics.

In our investigations we tapped bacteria directly from the floor of the cavity of clinical cases and, without further manipulations, dropped the scrapings into a sterile test tube, thus reducing the impending risk of eventual contamination.

In some cases of superficial caries, we also drilled a still deeper layer of sound dentin, in a matter of testing the reliability of our method. The broth inoculated with these scrapings proved sterile after 2 days of incubation. A conclusion logically followed that the invasion seems to be utterly limited; if we drill a little deeper, we enter a sterile zone. On the other hand, if we do not clean out the area "surgically," we risk contamination by secondary invaders. In fact, we "sinned" in both directions; for a certain number of our

cases proved sterile, while others showed mixed cultures. This seems to be inevitable, since we deal with "outposts," with "pioneer" bacteria, and their invasion into hard dental structures is essentially limited to a narrow zone of demarcation.

It is significant that in most of our cases, streptococci were the only organisms obtained in pure culture. All the other bacteria, occasionally isolated, such as micrococci, gram-negative bacilli, filamentous bacteria, and others, were obtained in mixed cultures. We should like to point out that most of our pure streptococci cultures were isolated from cases of superficial enamel caries, with discoloration as the only macroscopic sign of an initial attack.

With deeper cavities, there is always the risk of contamination, and we are tempted to speculate that some of the mixed cultures of our dentin scrapings could be thus explained as contamination. Further extension of research along the present lines, with a more refined technic, might bring us closer to the understanding of the role of bacteria in the etiology of caries.

Our experiments conducted over a 4-year period were performed at all kinds of weather and seasonal variations. The scrapings from both "sound" enamel and dentin yielded, in a great percentage of cases, streptococci in pure culture. The long-debated question[11] whether organisms are present in advance of the soft dentin, or softening precedes invasion, could be dismissed as irrelevant, with the use of our rigorous technic of a thorough removal of the softened dentin. In all our positive cases, bacteria were present in advance of the soft dentin, and in order to get the scrapings we had to discard the excavator and drill with the engine. It is not excluded, however, that some other kind of alteration, chemical or physical, preceded invasion.

In conclusion, our material seems to show that the main invaders are streptococci. We could not identify them, however, with any particular subgroup or species, although a fair percentage of them seem to belong to the viridans group and may be related serologically.

SUMMARY

The subject of this paper is the description of methods in caries investigation devised to verify the fact of bacterial invasion. The methods employed were: (a) tapping bacteria directly from clinical cases in routine preparation of cavities, and (b) identification of the organisms by cultural, morphological, biochemical, and serological methods.

Our results have shown that the organisms most frequently found in the floor of the cavities of initial caries are microaerophilic streptococci not fully identified as yet.

Our broader goal, the determination of bacterial identity by antigenic analysis, was not achieved with the limited material on hand.

The authors are greatly indebted to Dr. R. E. O. Williams of the Streptococcal Reference Laboratory, London, for his kindness in typing 23 of the strains. The authors wish also to thank Professors N. Grossowicz and A. L. Olitzki of the Department of Bacteriology, Hebrew University—Hadassah Medical School, for their interest and helpful discussion.

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TIME COURSE OF PAROTID SECRETION IN MAN

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ATTEMPTS, in recent years, to delineate mechanisms involved in secretion of specific salivary constituents, such as sodium, potassium, and small organic molecules, have leaned heavily on observed relationships between rate of fluid secretion and concentration of the specific constituent in the saliva.[1-3] Determination of the exact nature of the relationship in each case is, of course, dependent on availability of acceptable measurements of fluid flow rate. This, as reported by Becks,[4] may vary 100 per cent or more in man, even in short consecutive periods of collection under conditions of little exogenous stimulation. A recent report by Hildes and Ferguson,[2] concerning secretion of fluid and ions by stimulated parotid gland, indicates that variation in fluid secretion rate of human subjects may be even more frequent and extensive than formerly indicated. Because of the possible importance of such an effect on consideration of mechanisms of secretion, this investigation of the time course of parotid gland secretion, under further simplified conditions of reduced exogenous stimulation, was undertaken.

METHOD

Young adult human subjects (10 male and 1 female) served as saliva donors. Subjects were not fasted but collections followed the meal period by at least one hour. Just prior to, and during, the saliva collection, subjects were comfortably seated and extraneous stimulation was kept to a minimum.

Conventional parotid cups[5] were used to obtain the secretion. Measurements of secretory flow were made by two methods. In the first method (I), secretion was led by rubber tube from the collection chamber of the parotid cup to a small capacity (generally 0.2 ml., calibrated in steps of 0.01 ml.) serological pipette. Volumes were recorded at intervals of several seconds, as timed by a stop watch.

The second method (II) for recording was devised for this investigation and involved the use of a flexible steel strip clamped to a fixed support at one end but left hanging free at the other. A light metal pan was affixed to the free end of the steel strip and a celluloid pointer was attached to this so that deflections of the free end of the assembly, resulting from dropwise addition of saliva from the collecting tube to the pan, could be recorded by

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contact against a revolving smoked kymograph drum. The recording device was calibrated by addition of known weights to the collection pan; time intervals were marked directly on the kymograph record.

RESULTS

The rate of secretion from one parotid gland of each of 11 subjects was measured, by either of two methods already described, as a virtually continuous function of time. The results are indicated, in a form simplified to facilitate analysis, in Table I. More complete data for 4 of these subjects are given graphically in Fig. 1.

Table I shows, for a total collection period of 4 to 10 minutes, the per cent of the total secretory flow obtained at intervals of 20, 40, 60, and 80 per cent of total collection time. At 100 per cent time, of course, 100 per cent of the total flow was recorded. As shown in Table I, one group of subjects (Nos. 1-3) displayed a time course of secretory flow which was essentially linear. The main bulk of the subject group, however, displayed cumulative secretion flow values which indicate marked departure from constancy during the 4- to 10-minute total time of collection. These two general trends of flow behavior were observed with both methods for flow recording (Table I).

TABLE I
CUMULATIVE VOLUME* OF PAROTID SECRETION AT SELECTED INTERVALS OF TOTAL COLLECTION PERIOD (TCP)

SUBJECT	% OF TOTAL SECRETION				TOTAL SECRETION (ML. OR GM.)	TOTAL TIME (MIN.)
	20 % TCP	40 % TCP	60 % TCP	80 % TCP		
1	19	38	61	77	.172†	4.0
2	28	48	63	78	.600	4.0
3	29	46	66	89	.350	10.0
4	16	30	52	74	.950	8.0
5	10	25	63	88	.200	10.0
6	19	39	45	88	.200	10.0
7	21	33	70	94	.300	10.0
8	28	55	74	89	.065†	10.0
9	35	49	91	97	.159†	10.0
10	30	40	58	80	.250	10.0
11	8	36	52	76	.445	10.0

*Cumulative volumes are expressed as per cent of total secretion for the indicated period.
†Secretion collected by Method I and expressed as milliliters.

Closer examination of the course of the volume flow of parotid secretion with time is permitted by the graphic presentation in Fig. 1 which shows cumulative flow for consecutive brief intervals during the total collection period for Subjects 1, 5, 3, and 9 of Table I. These cases displayed time courses of secretory flow that may be representative of distinct flow patterns.

The first possibly specific pattern of secretory flow is represented by the time course shown in Fig. 1, *A*. In this case, cumulative flow values show a generally linear trend with time and an absence of periods of drastic change in secretion rate. The time courses of secretion for 4 discrete collection periods, two occurring consecutively on one day (*a*, *c*) and two on another, 8

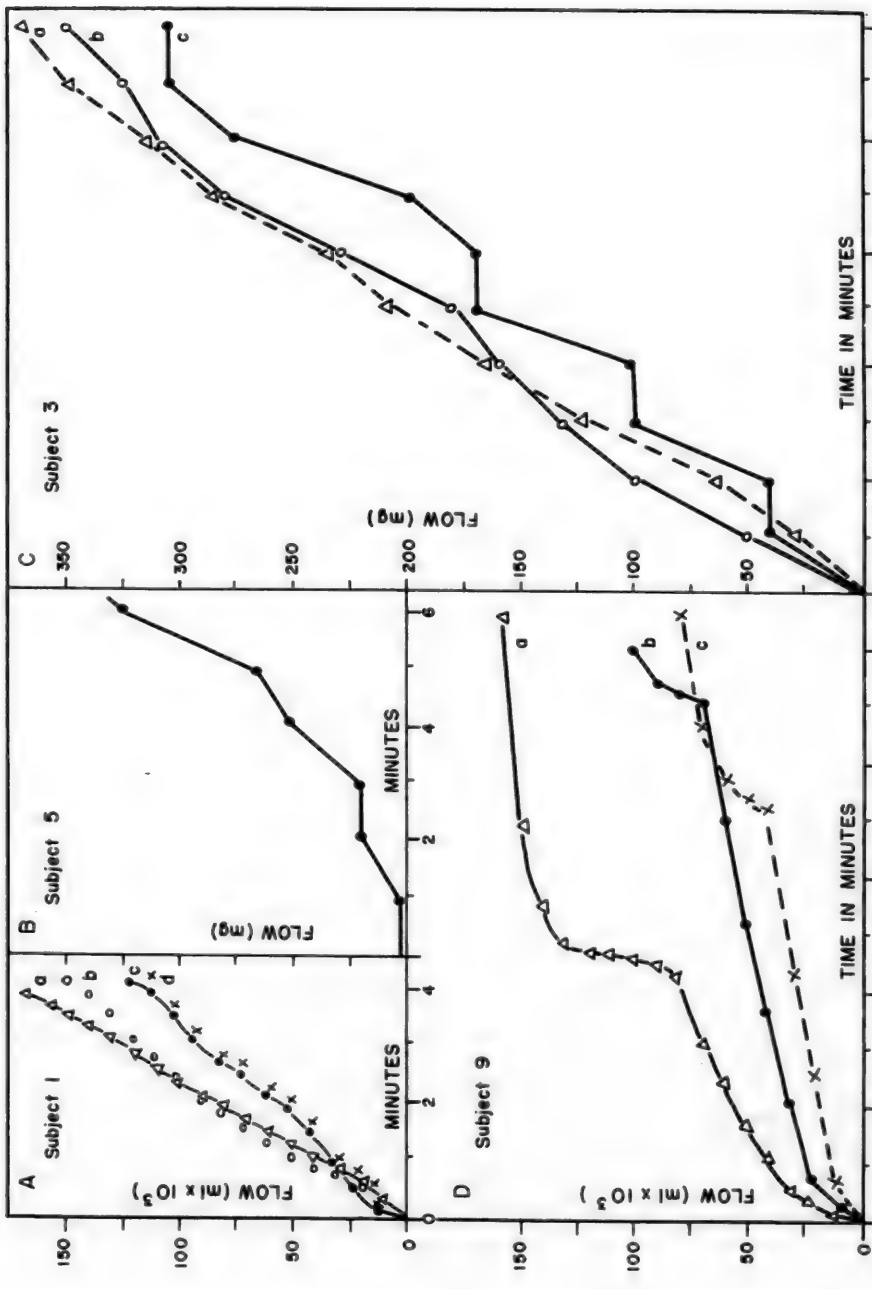

Fig. 1.—Time course of parotid secretory flow in 4 subjects. Multiple curves of Subjects 1, 3, and 9 represent separate collections. Rate of flow for Subjects 1 and 9 determined by Method I; for Subjects 5 and 3 by Method II.

days later, but all with the same subject, are also shown here. Although some departure from linearity is observed especially for curves *c* and *d*, a generally linear trend and an absence of periodic large changes in rate is characteristic of each of these.

A second possibly representative pattern is one of periodic rapid rate fluctuations. The over-all cumulative flow may show a nonlinear general course (Fig. 1, *B*), or a generally linear over-all tendency (Fig. 1, *C*). When the linear tendency is displayed, distinction between this and the first pattern may be somewhat arbitrary and depends on the degree of periodicity displayed during the time course of secretion. In this case also (Fig. 1, *C*), the results show a tendency for a particular secretion pattern to be generally characteristic for a specific subject. This is evidenced by the similar time course curves obtained in 3 collection periods on two different days (only collections corresponding to curves *a* and *c* of Fig. 1, *C* were made on the same day).

A third pattern which may possibly be delineated is one in which longer periods of relatively linear response alternate with shorter periods of marked change in secretion rate. This is exemplified by Fig. 1, *D* which shows 3 time course curves derived from collection data obtained on 3 separate occasions, one to 2 weeks apart, from the same subject. In this case, also, repeated observation has revealed a seemingly constant pattern of secretion rate change during a total rate measurement period of 10 minutes or less (Fig. 1, *D*).

DISCUSSION

The data presented show clearly that the rate of parotid secretion, when observed at frequent intervals during a 4- to 10-minute total collection period, may vary markedly during this time. The character, course, and reproducibility of these variations seem to justify two tentative, preliminary generalizations. First, even though a degree of overlap is observed, the data suggest that there may be 3 general patterns of time-dependent change in secretory rate. These involve (1) relative absence of marked rate change during the collection period, (2) rapid, large fluctuations in rate generally conferring a degree of periodicity on the time course of secretion and resulting in either a nonlinear or linear over-all time course of cumulative flow, and (3) alternation of longer periods of relatively linear response with briefer periods of marked rate change leading, in this case, to a periodic rate change which is generally nonlinear in its time course.

The second generalization suggested by the data concerns the constancy of the rate pattern in any selected individual. The available data lead to the impression that, under constant conditions, rate patterns for each individual show recognizable similarity. Further work is, however, required to confirm and further substantiate this; the data are too limited to consider this relationship firmly established.

Two methods of collection and measurement of cumulative secretion rates have been employed. Since patterns of secretion obtained by both methods

appear comparable and since the pattern most frequently found in these experiments (i.e., rate fluctuation showing periodicity) agrees generally with the record obtained by Hildes and Ferguson by still a third method,[2] it appears that the observations reported here are not artifacts attributable to methodology.

The mechanism of the rate changes reported here and also by Hildes and Ferguson is unclear and requires further investigation for delineation. Overt muscle contraction associated with deglutition has been carefully noted in the present study, but no correlation of such activity, or of respiratory movements, with the observed changes in secretion rate has been found. It is also of interest that the observations of secretory rate fluctuation of Hildes and Ferguson were made using stimulated conditions; our observations indicate similar rate variation occurs under "resting" conditions. Nonetheless, it may reasonably be speculated that periodic or aperiodic bursts of activity in gland secretory or myoepithelial elements may account for the observations reported. Further investigation is, however, required to test this possibility as well as the possible influence of the brief-duration secretion-rate changes on assumed rate-dependent processes.

SUMMARY

Rate of secretion by one parotid gland of each of 11 subjects has been observed at frequent intervals during a 4- to 10-minute period of saliva collection. Two methods of recording cumulative secretory flow were employed. Three general patterns of flow rate change were observed: (1) flow rate may remain relatively constant during the total collection period; (2) flow rate may show rapid periodic fluctuation; or (3) rate of secretion may display slow periodic fluctuation. The data, although limited, suggest that the time pattern of secretory flow is characteristic for the individual subject.

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STUDIES ON SALIVARIADENECTOMIZED HUNT-HOPPERT CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS

II. Influence of Salivariadenectomy on Rate of Food Consumption

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A PREVIOUS study showed that salivariadenectomy decreased the time for initiation of caries in susceptible rats slightly, but markedly decreased this time in resistant rats.[1] This was consistent with previous results that salivary flow was inversely related to caries incidence. Observations of salivariadenectomized rats indicated that they were taking longer to consume food and were eating more frequently than control rats. Therefore, it is conceivable that caries might have developed faster in salivariadenectomized rats due to the change in mode of feeding rather than the deprivation of saliva. This seemed important enough to justify a quantitative study, even if the method used lacked precision. This study was designed to determine the influence of salivariadenectomy on the rate of food consumption in the rat.

EXPERIMENTAL

The feeding time was observed on two sets of experimental rats at either 2 weeks or 6 weeks after salivariadenectomy. The data for the observations at 2 weeks and 6 weeks after salivariadenectomy were combined because there was no mean difference between the two groups and previous experiments had shown that operated animals recover their ability to consume as much food as control rats about 4 days after surgery.

The method of salivariadenectomy has been given in a previous report.[1] The groups of animals are listed in Table I. *In the first experiment,* a group of normal rats (Control 1) was compared to littermates of completely salivariadenectomized rats. *In the second experiment,* a group of normal rats (Control 2) was compared to littermates which either had a portion of the parotid ducts removed and the severed ends ligated, or had the submaxillary and sublingual glands excised. The completely salivariadenectomized rats and those in Control 1 were observed over a continuous 24-hour period whereas the animals in the remaining groups were observed for a 6-hour interval from 9:00 P.M. to 3:00 A.M. During these 6 hours the rats consumed about 40 per cent of their daily food.

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For the actual observation period, the racks containing the rats in wire-bottom cages were placed so that one person could observe about 30 rats by scanning each row of cages. A notation was made each time an animal was observed consuming food. Each notation was treated as one minute of feeding time. If an animal consumed food continuously, a notation was made approximately every minute. The rate of food consumption for each rat was determined by totaling the number of notations for that animal and dividing by the amount of food consumed. The term, *rate of food consumption*, designates time required to consume a unit of food instead of amount of food consumed in unit time. All of the rats observed in this experiment were given food and water ad libitum.

RESULTS AND DISCUSSION

The data are given in Tables I and II. The mean time required by rats in Control 1 to consume a gram of food was 4.7 ± 0.37 minutes. Their completely salivariadenectomized littermates consumed a gram of food in 13.8 ± 2.72 minutes. The difference between these groups is statistically significant.

TABLE I

EFFECT OF SALIVARIADENECTOMY ON RATE OF FOOD CONSUMPTION

GROUP	NUMBER OF RATS	MEAN TIME IN MINUTES REQUIRED FOR CONSUMPTION OF ONE GRAM OF FOOD, WITH STANDARD ERROR
Control 1 (normal rats)	15	4.7 ± 0.37
Submaxillary and sublingual glands and parotid ducts removed (completely salivariadenectomized)	29	13.8 ± 2.72
Control 2 (normal rats)	17	6.3 ± 0.83
Parotid ducts removed	23	6.8 ± 0.52
Submaxillary and sublingual glands removed	16	10.1 ± 0.88

TABLE II

STATISTICAL COMPARISON OF RESULTS OF EFFECT OF SALIVARIADENECTOMY ON RATE OF FOOD CONSUMPTION

COMPARISON	DIFFERENCE	"t" VALUE	LEVEL OF SIGNIFICANCE
Control 1 with submaxillary and sublingual glands and parotid ducts removed	9.1 ± 2.74	3.32	< 0.001
Control 2 with submaxillary and sublingual glands removed	3.8 ± 1.21	3.14	< 0.001
Control 2 with parotid ducts removed	0.5 ± 0.98	0.51	> 0.5
Submaxillary and sublingual glands removed with parotid ducts removed	3.3 ± 1.02	3.24	< 0.001
Control 1 with Control 2	1.6 ± 0.91	1.76	> 0.1

Rats in Control 2 consumed a gram of food in 6.3 ± 0.83 minutes. Although the rate of food consumption for rats in this control group is greater than for rats in the first control group, the difference in rates of food consumption is not statistically significant. Animals with only parotid ducts removed did not show a significant increase in rate of food consumption over littermate controls, whereas those with submaxillary and sublingual glands excised showed a significant increase.

Rats which were completely salivariadenectomized showed the highest rate of food consumption. The next highest rate of food consumption was shown by rats which had the submaxillary and sublingual glands removed. Rats with only the parotid ducts removed showed a rate of food consumption slightly greater than control rats. Studies now in progress reveal that the submaxillary and sublingual glands contribute about 75 per cent of the saliva in rats. Therefore, these data suggested that the rate of food consumption is inversely related to the amount of saliva.

SUMMARY

Completely salivariadenectomized rats required more time than normal control rats to consume the same quantity of food. Rats with submaxillary and sublingual glands extirpated also spent more time in feeding than controls. Rats with parotid ducts removed took only slightly longer than controls to consume the same amount of food. Apparently, there is an inverse relationship between the amount of saliva, and time required to consume food.

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ELECTRON MICROSCOPY OF DEVELOPING ENAMEL MATRIX IN THE SYRIAN HAMSTER

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FEW electron microscopic studies of developing enamel have been carried out, and contradictory findings have been reported. Sognnaes, Scott, Ussing, and Wycoff[1] described an early homogeneous matrix which becomes fibrillar, and Watson and Avery[2] have found a fibrillar matrix developing from the cytoplasm of the ameloblast. These latter authors found no evidence of interrod substance, while Sognnaes and his co-workers were able to describe this structure in developing teeth.

Watson and Avery felt that from their evidence some changes in nomenclature were advisable. Since the term "prism" implied plane sides and the suggestion of a single crystal, this was dropped in favor of enamel rod. The rod was shown to consist of two parts, an inner rod "core" and an outer "rod wall." "Interprismatic" or "interrod" was discarded because no substance could be found between the rods. "Rod" or "prism sheath" was also discarded, since the wall was found not to surround the core completely.

Recently, Nylen and Scott[3] have reported in abstract form on the developing incisor in mice. They found that in early formed ameloblasts the mitochondria were situated basal to the ameloblast nucleus, and that a band of dense fibrillar material separated them from the cell membranes. The first formed matrix was composed of very fine fibrils.

As part of a larger study of tooth structure, developing enamel from the incisors and molars of hamsters was examined by electron microscopy. This report deals with the formation of enamel matrix with special regard to its origin, arrangement, and structure, and changes taking place as the tissue becomes older.

MATERIALS AND METHODS

Developing enamel matrix was obtained from the molar teeth of newborn hamsters, and the basal end of the mandibular incisors of fully grown hamsters. The hamster was chosen for the following reasons: (1) the ease with which the developing molars could be dissected out, (2) the histologic similarity of these molars when fully formed to that of human beings, (3) the widespread use of the hamster in dental research, and (4) at the apical end of the incisor, enamel continues to be formed throughout the life of the animal.

Under a dissecting microscope, vertical incisions were made in the molar areas of newborn hamsters. The tooth germs were identified and 1 per cent

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buffered osmium tetroxide (pH 7.2) poured on the tissue. After 10 minutes, the tooth germs were carefully removed, cut into small pieces and further fixed for 2 hours, rinsed in water for half an hour, and dehydrated in increasing strengths of ethyl alcohol. The tissue was then embedded in methacrylate and sectioned on a Porter-Blum microtome at 200 to 300 Å. The incisor enamel was obtained by anesthetizing the animals with intraperitoneal Nembutal and carefully splitting the bone of the mandible to reveal the growing end of the incisor tooth. Osmic acid was poured on as before and the basal 4 to 6 mm. of the tooth removed. Further treatment was as for the molars.

Electron micrographs were taken with Philips E.M.U., 100 Å at 60 kv.

No decalcification or shadow casting technics were used, since many objections can be raised regarding preparation methods, which were therefore kept as simple as possible.

RESULTS

Because of certain similarities of these results with those of Watson and Avery,[2] their suggested nomenclature will be used with certain exceptions. While agreeing with the idea of Watson and Avery of a wall forming peripherally while the "core" lags somewhat in development, it is felt that the core should be considered a "rod" and therefore the wall would be "interrod." The findings in the molars of the hamster will be described first.

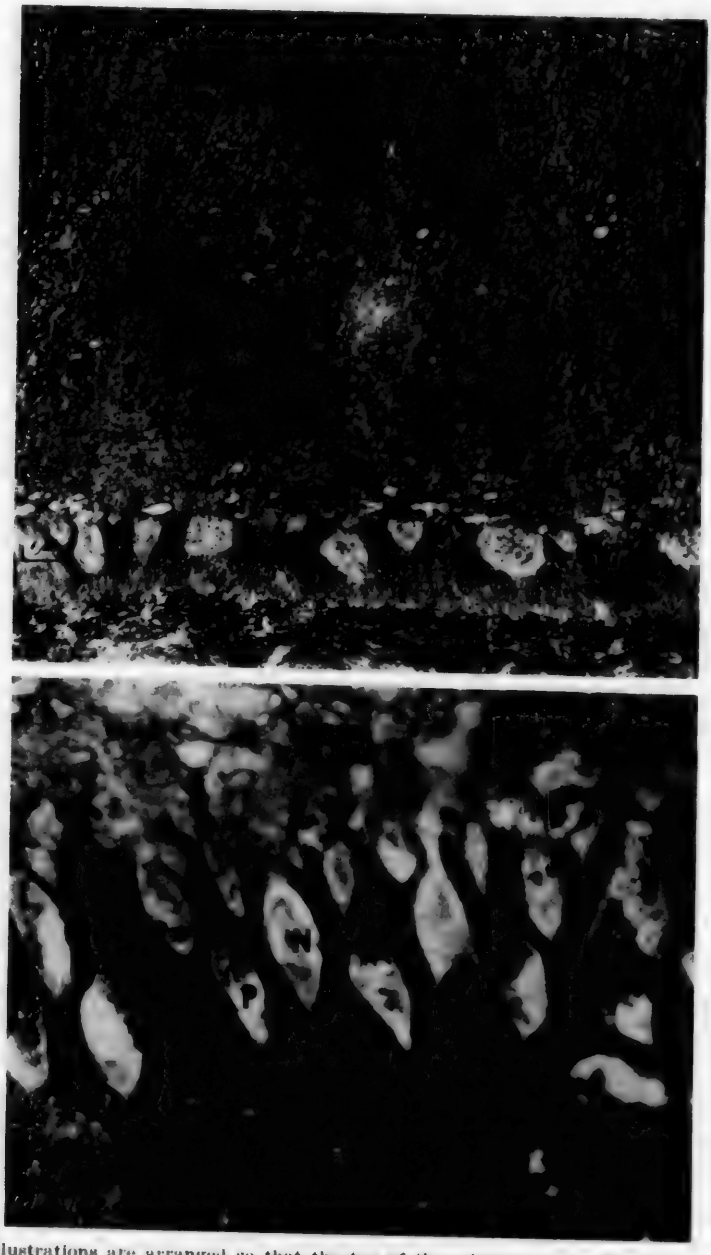
The first formed enamel matrix is fibrillar in character, and arises directly from the ameloblast cytoplasm. No homogeneous precursor of the matrix could be found, no terminal bar apparatus was seen, and cell membranes could not be found between the ameloblasts near the forming enamel matrix (Figs. 1, 2, 3). However, an extension of ameloblast cytoplasm, presumably Tomes's process as seen in light microscopy, protrudes some distance into this first formed matrix. These extensions do not contain endoplasmic reticulum but dark osmophilic granules can be seen (Figs. 1 and 2).

At a slightly later stage, as the ameloblast cell body retreats, this extension or core of cytoplasm becomes fibrillar, the fibrillation always commencing on the same side of the core, and continuing until the core is completely filled. Fig. 2 is a slightly oblique section showing a row of fully fibrillated cores, succeeded by a partially filled row, and a nonfibrillated row as the cytoplasm of the ameloblast is approached. Fig. 3 is a higher magnification of part of Fig. 2, showing the abrupt origin of the fibrils and the continuation of the cytoplasm into the forming matrix as the core or rod.

The columns of fibrillar material which separate the cores blend with each other and it is impossible to find a dividing line between them (Fig. 2).

The fibrillation of the cores in a definite pattern is shown in Fig. 4. Whereas the first formed interrod matrix is more or less at right angles to the long axis of the core, the core fibers are parallel to this axis. No structure identifiable as the rod sheath was found in any of the molar sections.

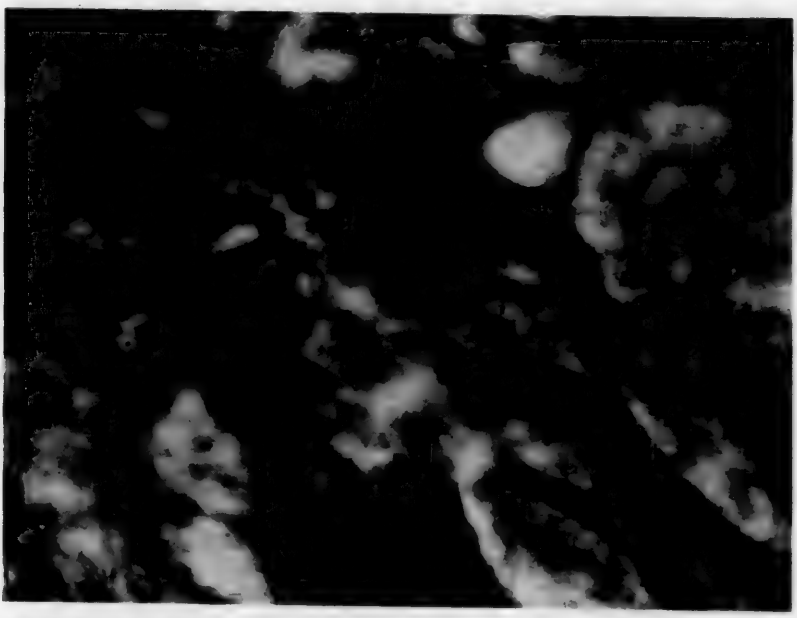
The fixation of incisor tissue was better than that of the molars, particularly in regard to the cytoplasm. This can be accounted for by the fact that the older animals were still alive when the initial fixation was begun.



All illustrations are arranged so that the top of the micrograph is either (1) nearer the ameloblasts or, (2) in the case of the incisors, nearer the apical growing end of the incisor tooth.

Fig. 1.—Section of developing hamster molar. Ameloblasts showing earliest formed matrix (*M*), developing from basal end of the cell nearest the dentin (*D*). No terminal bars or cell membrane can be found at the dentinal end of the ameloblasts.

Fig. 2.—Oblique section of developing molar enamel matrix. More matrix formed than Fig. 1. Nearest the dentin (*D*), rods are fully fibrillated (*F*). As ameloblast cytoplasm is approached, the next layer is only partially fibrillated (*P*), and then nonfibrillated (*N*). (Same magnification as Fig. 1.)



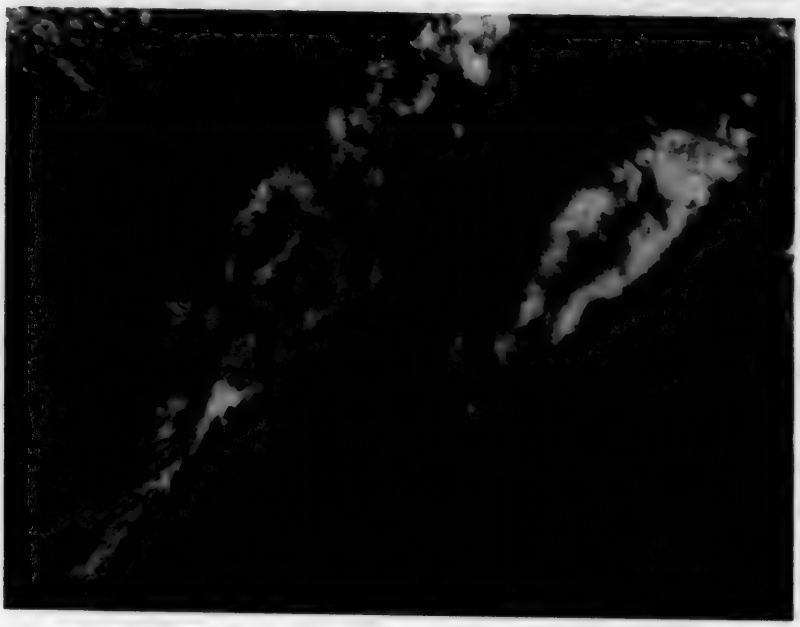


Fig. 3.—Higher magnification of Fig. 2, showing the abrupt origin of the fibrils.

Fig. 4.—Molar matrix showing the orientation of interrod fibrils (*I*) at right angles to core or rod fibrils (*R*). Progressively, from left to right, fibrillation is more complete.

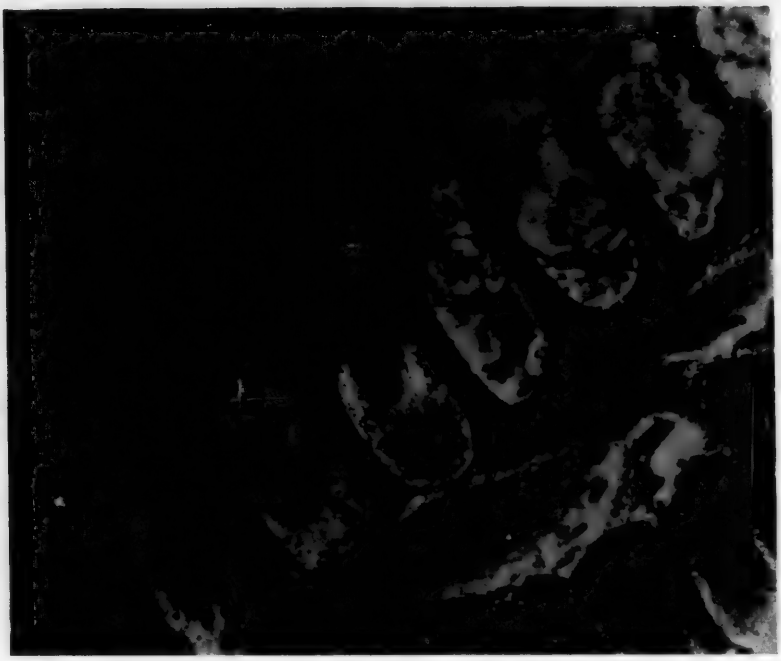


Fig. 5.—Developing hamster incisal enamel matrix. The younger enamel matrix consists of cytoplasm (*C*) surrounded by interrod fibrils (*I*). Alternate rows or sheets of rods lie at right angles to each other, each sheet being more fibrillated as the incisal edge is approached (toward incisal edge ↓).

Fig. 6.—Developing hamster incisor matrix. The cytoplasm communication of one sheet to another is illustrated (*B*). The progressive closure of this communication, and the fibrillation of the core or rod from this aspect is seen (toward incisal edge ↓).

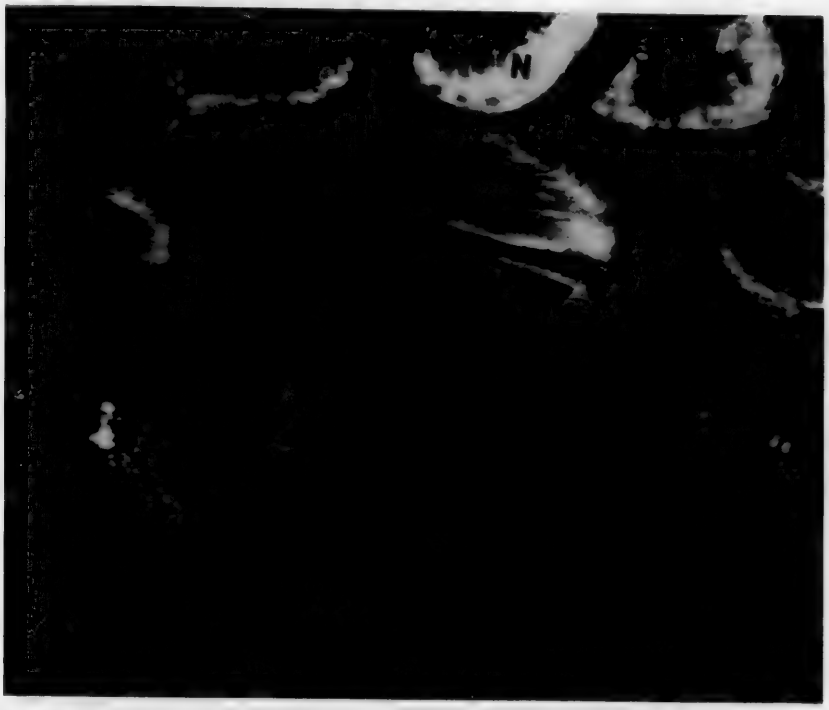


Fig. 7.—Older hamster incisor matrix. Not yet fully fibrillated rods (*N*), showing fibrillation beginning in each instance from same side of rod. Cross sections of rods fully fibrillated (*R*), cores lying between longitudinal sections.

Fig. 8.—Higher magnification of Fig 7. The interrod substance (*I*) is thicker between rods of the same sheet than between rods of adjacent sheets. The boundary between rod (*R*) and interrod (*I*) substance is now difficult to define, fibril orientation being only discerned with difficulty.

The incisor specimens bear out the findings in molar enamel. An added advantage in the incisor is that, as pointed out by Tomes,[6] the enamel rods in rodent incisors lie in alternate sheets, each adjacent sheet being at right angles to its neighbor. Thus, in an ideal section, cross sections of rods are seen between two longitudinal sections and vice versa.

Alternate layers of cross sections of rods between longitudinal ones show that the interrod matrix forms first (Figs. 5 and 6). The interrod material forms all around the as yet cytoplasmic rod except on one side. This open side, always on the same side of the core in a given sheet or layer of rods, eventually closes, but just prior to its completion fibrillation of the rod commences from the open side (Figs. 5 and 6). This internal transformation continues until the cytoplasm of the rod is completely filled with fibrils. Before completion of the open part of the interrod material, the cytoplasm of one layer of rods is continuous with that of the adjacent layer.

When the rods are fully fibrillated, the only difference between rod and interrod substance is the direction of the fibrils. In the incisor the interrod substance is thicker between two rods of the same sheet than between rods of adjacent sheets (Figs. 7 and 8).

In general, the incisors show the same features as the molar preparations, namely, a fibrillar interrod material formed in advance of the rods, which later also become fibrillar.

DISCUSSION

Electron microscope studies of adult enamel have failed to agree on the presence of interrod substance. Bernick, Baker, Rutherford, and Warren[4] reported none, while Takuma, Suzuki, Osawa, and Tsuchikura[5] found abundant amounts on one side of the rod and total absence on the other. However, as these studies were carried out with demineralized sections, the action of the acid used would be expected to have some effect on the delicate submicroscopic fibrils. In this study, enamel "rods" are used to denote the portion of enamel which is formed from the cytoplasmic core and, developmentally at least, an interrod substance is present.

Watson and Avery[2] used the term "wall" for what is considered here interrod. However, they felt that one ameloblast was responsible for one wall and one core, or, in the terminology of this paper, the interrod substance on one side of a rod, and the rod itself. The present findings do not agree with this concept. Instead, the interrod fibrils were found to mingle with one another, so that the impression was formed that the interrod substance was a continuous network or stroma, in which the rods were formed secondarily. This idea is not entirely new. Mummery[7] stated that processes of the ameloblasts divide into bundles and interlace, to form the greater part of the enamel matrix, the scaffolding of each rod being therefore the product of several ameloblasts and not of one cell only.

The evidence is strongly in favor of enamel matrix arising by a transformation of, rather than a secretion by, the ameloblasts. The failure to find cell walls and terminal bars also suggests a syncytial nature of the basal portion

of the ameloblasts, certain areas continuous with one another becoming fibrillar and forming the interrod fibrils, and, as the ameloblasts retreat, the cytoplasmic projections progressively forming into rods. Such an explanation would help solve many problems in enamel histology. The tortuous course of the fully formed enamel rods would not necessarily have to be accounted for by movements of the ameloblasts themselves, and the increased area of the enamel at the tooth surface from that at the enamel-dentinal junction may result from the increase in the number of cytoplasmic projections as the enamel thickens, without the increase in the number of cells. This would result in supplemental rods, as suggested by Mummery,[7] although the existence of such rods is denied by those who explain the difference in area by a gradual taper of the rods as they proceed outward.[8] At the magnifications shown here, even the slightest taper of the rods from the enamel-dentinal junction to the surface would be evident, and no evidence of this has been found.

Rod sheaths have not been found in this study. This does not preclude their existence in adult enamel since they may result from condensation of the submicroscopic fibrillar matrix at the periphery of the rods during calcification. On the other hand, they may be artifacts caused by acid treatment during decalcification. Further work is planned to determine the presence and nature of the rod sheath.

SUMMARY

Electron micrographs of developing teeth of hamsters show that:

1. Enamel matrix develops intracellularly and in two phases.

2. The first formed matrix is the interrod material.

3. The rods develop from extensions of the ameloblasts and at first exist as cytoplasm enclosed by the interrod substance. The transformation of the cytoplasm into fibrillar material starts on one side of the rod. The fiber direction is well organized. Those of the rod run in its long axis while those of the interrod material are at right angles to the rod.

4. No rod sheath is found during developmental stages.

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A METHOD OF MEASURING THE ADHESION OF FOODSTUFFS TO TOOTH SURFACES

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BIBBY and his associates[1, 2] have reported that foodstuffs vary considerably in their clearance from the mouth, an important factor apparently being the adhesiveness of the foodstuff. Lundqvist[3] has also shown that the rate of oral clearance of carbohydrate foods varies widely, depending again on the physical properties of the foodstuff. As yet, very few investigations have been concerned with the direct adhesiveness of foodstuff to tooth surface and, as indicated by Nevin,[4] discussion regarding this topic has generally remained qualitative or subjective.

This investigation is concerned with the measurement of the phenomenon of adhesion of foodstuffs to teeth, using as a measure of adhesion the force required to break the bond between the foodstuff and the tooth surface. It is emphasized that other properties of the foodstuff such as solubility, permeability, "packing quality,"[4] "pushability,"[5] etc., may be of as much importance as adhesiveness in determining which foods will be retained around the teeth. This multiplicity of factors underlies the importance of assessing each factor separately, so that an understanding of the whole will become possible.

EXPERIMENTAL PROCEDURES

The measurement of adhesion is problematical and is valueless if it is not clear what is meant by adhesion. In the printing industry, for example, adhesion usually refers to tackiness, while in the formulation of industrial glues may different criteria are used to evaluate adhesion, depending on the use to which the material will be put. Adhesion in the present investigation is defined as the force which holds the foodstuff against the tooth surface. Thus, an attempt is made to measure this bonding effect by measuring the force required to break the bond between a foodstuff and a tooth surface.

Preparation of Tooth and Metal Surfaces.—It was not possible to measure adhesion on intact tooth surfaces because they are irregular, rough, and corrugated. Such surfaces vary from tooth to tooth and would be difficult to evaluate. In any case, "specific adhesion" caused by molecular forces of attraction

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on dense surfaces, such as crystal surfaces, is now considered to be the main type of adhesion which occurs. According to De Bruyne and Houwink,[6] the mechanical "locking" effect which would vary on rough surfaces is not so important as was previously considered. For these reasons, the crowns of caries-free, extracted, human, central incisor teeth were polished to expose a flat area of enamel for test purposes. The labial surfaces of several teeth were ground flat on a rough carborundum wheel and the teeth embedded in quick curing acrylic resin or Selectron 5119* with the flattened labial surfaces level. Polishing was completed using increasingly fine abrasive papers.† All polishing procedures, except the brief final polish with emery paper, were done with the paper and the tooth wet. No dentin was exposed or the tooth was discarded. The average area of flat enamel available was approximately 0.05 sq.in. Pairs of enamel surfaces were then trimmed to the same surface area with sandpaper discs.

The polished teeth were prepared for mounting in the adhesion tester as shown in Fig. 1, *A*. Two brass holders were fashioned and the polished tooth surfaces *(1)* fixed to the brass holders with quick curing acrylic resin *(2)*. Two tapering pins *(3)* were used to guide the surfaces together accurately. The brass holders were then fixed in the clamps of the adhesion tester as in Fig. 1, *B*. On the left of Fig. 1, *B* is a fixed clamp *(4)* with a pulley wheel *(5)*. On the right is seen a clamp *(6)* which is attached to the end of a trolley. The tooth surfaces *(1)* are pressed together by a weight *(10,* Fig. 1, *C)* hanging from a cord which runs over the pulley *(5)* and is attached to the clamp *(6)*. The test food is placed between the two tooth surfaces and pressure applied with the correct weight needed to exert a force of 75 lb./sq.in. for 60 seconds. Each pair of tooth surfaces has a different area, so it is necessary to measure this area of contact accurately and to calculate the actual loading weight necessary to apply the standard 75 lb./sq.in. which is used to squeeze the test food between the enamel surfaces.

The machine used for the adhesion tests is the Serigraph.‡ This machine, as shown in Fig. 1, *C* and *D*, is a Scott I.P.-4 tensile strength tester. It consists of a long rigid plane *(8)* on which rests the trolley *(7)*. The test material is attached between one end of the trolley and the fixed clamp *(4)* on the plane. To test the tensile strength of the adhesion bond between the food and the tooth surfaces, the plane can be tilted as shown in Fig. 1, *D* and, as the plane inclines, the trolley tends to run down the sloping surface. Since the applied weight of the trolley increases with the steepness of the angle, eventually the material is broken and the trolley runs down the plane. As it does so the breaking strength of the food-tooth bond is automatically recorded on a calibrated chart *(9)*. In the present work, a 1 lb. trolley was used, and the rate of loading was 0.4 inch per second.

*Pittsburgh Plate Glass Co., Pittsburgh, Pa.

†Tufback, Durite Waterproof Paper No. 120-C, 180-C, 240-A, 320-A, 400-A, 600-A, and Emery Polishing Paper No. 0 from Behr-Manning Co., Troy, N. Y.

‡Scott Testers (Southern), Inc., 101 Blackstone Street, Providence, R. I.

Gustafsson[8] has made measurements of the adhesion of food preparations to stainless steel plates. If there is no difference between values obtained on human teeth and metal surfaces, it would be much more convenient to use a metal surface. To evaluate the importance of this factor, different metal and enamel surfaces were polished in the same way as the human tooth enamel. Brass, copper, stainless steel, human tooth enamel, and cow tooth enamel (labial surfaces of permanent lower anteriors) were compared, using a mixture of 200 mg. of corn flakes (grit size 20-30) to 0.1 ml. of water.

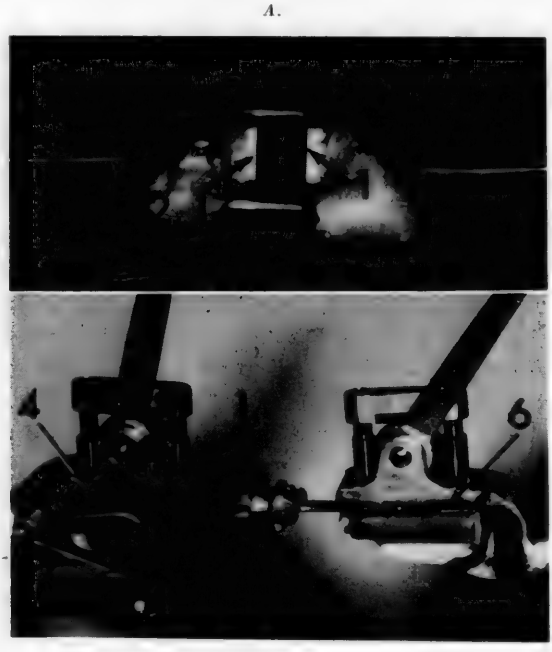
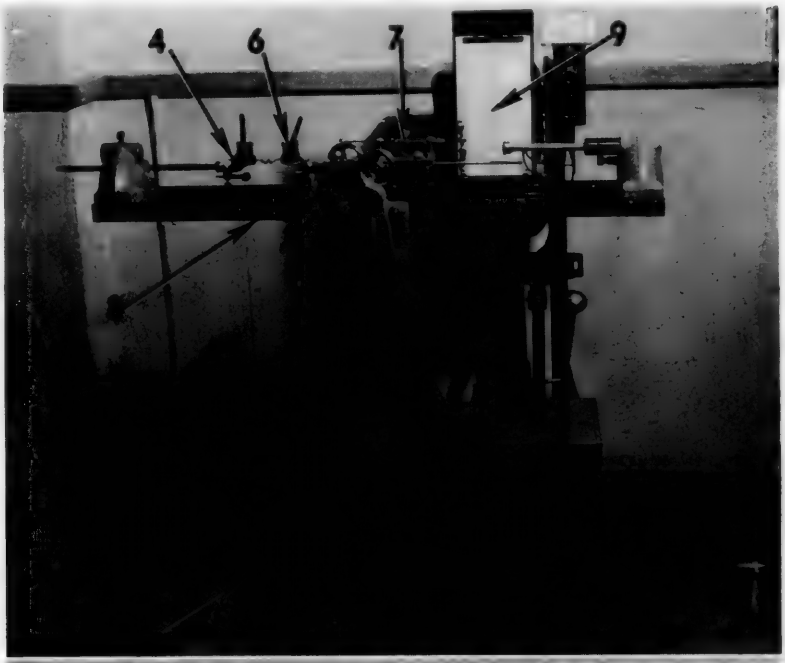


Fig. 1.—*A*, Polished tooth surfaces (*1*) mounted in acrylic resin (*2*) with steel guide pins (*3*).
B, Tooth surfaces (*1*) in fixed clamp (*4*) and trolley clamp (*6*). Pulley wheel (*5*).

Food Testing.—

A. *In vivo materials:* It was considered of interest to develop a method to measure the adhesiveness of foodstuffs immediately after they have been chewed in the mouth. It would be best to measure adhesiveness intraorally, but this has not been possible. A compromise was made by having the subject eat a portion of the food in the customary way and after the main bolus of food had been swallowed and after chewing movements of the tongue and cheeks were completed, the food adhering to the tooth surfaces (mainly lower posterior occlusal surfaces) was rapidly scraped off the teeth with dental instruments. This food-saliva sample was immediately placed between the test enamel surfaces mounted in the Serigraph, and the adhesion recorded as described. To compensate for variations in this technic each food was tested ten times on each



C.

D.

Fig. 1.—Cont'd. ***C,*** **Adhesion tester with inclinable plane (*8*). Fixed clamp (*4*), trolley clamp (*6*) and trolley (*7*). Calibrated chart (*9*) and weight (*10*).**

D, **Plane (*8*) inclined with trolley (*7*) at lower end of plane after food-tooth bond has broken.**

subject and the adhesion values averaged and the standard deviation calculated. Between each measurement the tooth surfaces are washed with water and ether and air dried. All experimental procedures, including the collection of the foodstuff from the mouth, are conducted in a constant temperature and humidity room (70° F., 65 per cent R.H.).

B. *In vitro materials:* The above technic is an in vivo procedure as far as the food preparation is concerned. The actual measurement of adhesion is an in vitro method. To test the accuracy of the method, weighed amounts of a foodstuff (corn flakes, grit size 20-30, U.S. Standard Sieve Series) were mixed with known quantities of the same subject's saliva (collected on ice 1 to 2 hours after eating[7]). Similar mixes were made with water and corn flakes. These were shaken for 30 seconds in a Wig-l-bug,* the resulting mixes being visually indistinguishable from the chewed foodstuff recovered from the mouth. Prior to weighing, the corn flakes were left for several hours to equilibrate with the atmosphere in the constant temperature and humidity room. Such test materials were immediately measured in the adhesion tester after the mechanical mixing.

RESULTS

A comparison of the adhesiveness of various foodstuffs will be reported later. The results in this communication are confined to an evaluation of the apparatus as a testing procedure.

Adhesion to Different Surfaces.—Table I records the values for corn flakes-water mixtures on different surfaces. There is little difference among the values for human teeth, cow teeth, and brass. Copper and stainless steel have clearly different values.

TABLE I

THE ADHESIVENESS OF CORN FLAKES-WATER MIXTURES* TO DIFFERENT SURFACES

Human teeth	11.3 ± 1.0
Cow teeth	12.3 ± 0.6
Brass	12.5 ± 1.5
Copper	14.4 ± 1.7
Stainless steel	7.3 ± 1.0

*0.1 ml. of water/200 mg. corn flakes. Values expressed in lb./sq.in. ± S.D.

Adhesiveness of In Vitro Mixtures.—Fig. 2 is a graph depicting adhesion plotted against dilution for mixes of corn flakes with water or saliva. These values are all of in vitro food mixtures mixed in a Wig-l-bug. Each point on the graph is the average of ten consecutive measurements and each point was made on different days. Repeat measurements at the same dilution were made in several instances. When the mixture was dilute, adhesion was low and as the mix became thicker it was more adhesive. After reaching a value of 10 to 11 lb./sq.in. (water-corn flakes), or 5 to 6 lb./sq.in. (saliva-corn flakes), the mix was too stiff to measure accurately in the tester, but it was apparent that adhesion fell off. When the mix was completely dry no adhesion was measurable.

*Crescent Dental Manufacturing Co., 1839 S. Pulaski Rd., Chicago 23, Ill.

Differences in adhesion values (10.2 ± 0.8 [S.D.] and 10.7 ± 0.9 [S.D.] lb./sq.in.) for two mixes of water and corn flakes at the same dilution (200 mg. per 0.10 ml.) were considered to be insignificant ($0.05 < P < 0.1$ – Student "t" test). The difference in adhesion values (10.2 ± 0.8 [S.D.] and 7.0 ± 1.3 [S.D.] lb./sq.in.) for low (200 mg. per 0.10 ml.) and high (200 mg. per 0.20 ml.) dilutions were considered to be significant ($0.001 < P < 0.005$ – Student "t" test). The lines drawn on the graph reveal that variations occur in adhesion values for corn flake preparations. These are thought to be due to differences in food from different packages, experimental error in the technic and, in the case of the saliva mixes, slight daily variations in saliva.

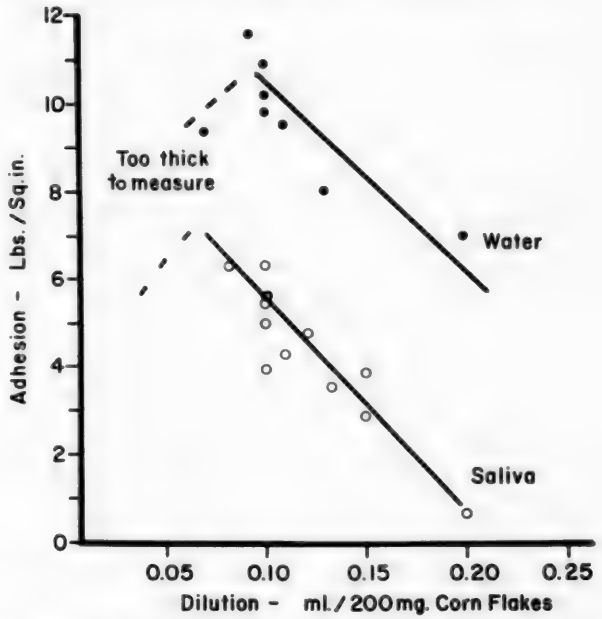


Fig. 2.—Comparison of the adhesiveness of mixtures of corn flakes made with water and saliva.

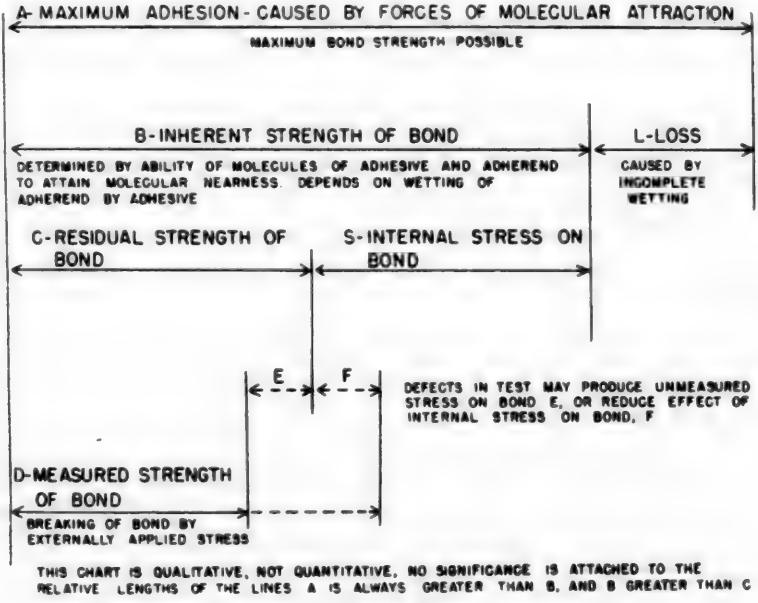
Although the saliva of the subject on different days may alter the adhesiveness of the food, the values followed a general trend fairly well. It was consistently demonstrated that the saliva mixes were less adhesive than the water mixes. Similar daily tests with different foods (caramel, bread, gingersnaps, etc.) supported the general conclusion that it is possible to differentiate between the adhesiveness of different foodstuffs mixed with water or saliva. In a later paper, a comparison of the adhesiveness of foods will be reported.*

*The measurement of the tensile strength of any bond must be conducted with certain controlled factors present. Careful examination of the ruptured bond should show a clean break between the adhesive and the surface. If a thin bond breaks so that the material is left partly on both surfaces this is acceptable only where the pattern of rupture shows that no cohesive failure of this bond has occurred. Such cohesive failure would leave material covering both surfaces, in part or completely. Cohesive failure was avoided in the present work by using thin bonds (2 to 7/1000 inch).

DISCUSSION

Any procedure for testing adhesion is debatable. This is, in part, due to the complex nature of the property of adhesion which in many instances cannot be reduced to a single measurement. Under different conditions and when differently defined, the reported value will change. Another problem concerns defects in mechanically testing adhesion bonds. This is illustrated in Fig. 3, wherein Reinhart[9] explains the discrepancy between the adhesion measured and maximum adhesion possible.

It has been clearly demonstrated that the adhesiveness of a food under different conditions can be compared (Table I and Fig. 2). Thus, the first objective has been realized and it is planned to use this type of apparatus to compare the adhesiveness of a variety of different foodstuffs to the tooth surface


A- MAXIMUM ADHESION - CAUSED BY FORCES OF MOLECULAR ATTRACTION
MAXIMUM BOND STRENGTH POSSIBLE
B-INHERENT STRENGTH OF BOND
DETERMINED BY ABILITY OF MOLECULES OF ADHESIVE AND ADHEREND TO ATTAIN MOLECULAR NEARNESS. DEPENDS ON WETTING OF ADHEREND BY ADHESIVE
L-LOSS
CAUSED BY INCOMPLETE WETTING
C-RESIDUAL STRENGTH OF BOND
S-INTERNAL STRESS ON BOND
E
F
DEFECTS IN TEST MAY PRODUCE UNMEASURED STRESS ON BOND E, OR REDUCE EFFECT OF INTERNAL STRESS ON BOND, F
D-MEASURED STRENGTH OF BOND
BREAKING OF BOND BY EXTERNALLY APPLIED STRESS
THIS CHART IS QUALITATIVE, NOT QUANTITATIVE, NO SIGNIFICANCE IS ATTACHED TO THE RELATIVE LENGTHS OF THE LINES A IS ALWAYS GREATER THAN B, AND B GREATER THAN C

—Relations between the factors involved in adhesion

Fig. 3. (From F. W. Reinhart, Survey of Adhesion and Types of Bonds Involved, John Wiley & Sons, Inc., 1954.)

under different conditions. Any treatments to the enamel surface with the object of reducing food adhesiveness can also be evaluated. At present, this work is being carried on and some results are reported in the accompanying paper.

Although no work has been done to measure the plasticity or "pushability" of foods, this apparatus could perhaps be modified for this purpose by placing the test food at the lower end of the trolley *(7)* and, as the plane *(8)* inclines, the resistance of the food to the applied weight could be recorded. This might be possible since the machine is not only calibrated to measure tensile strength in hundredths of a pound, but also to measure "stretch" in tenths of an inch. Incidentally, no "stretch" was observed in the adhesive bonds measured.

There are other testers available which would probably be even more accurate than the Scott I.P.-4 used in this investigation. The smaller Scott I.P.-2 tester or the newer testers employing strain gauges would be more suitable for measuring the low values which are recorded with breads and some other foods.

One valid objection to the test procedure is the routine use of 75 lb./sq.in. to press the food between the teeth. It is realized that the plasticity of different food mixtures will affect the thickness of the bond produced if a standard force is always used to press the teeth together. This means that it is not correct to assume that such bonds will have the same strength. The alternative is to standardize the bond thickness. In the present work this was not practicable because thick, easily standardized bonds often resulted in cohesive failure while the more satisfactory very thin bonds were not easily standardized in thickness. It was observed by using various foods removed from the mouth, that the bonds produced at 75 lb./sq.in. were of similar thickness (2 to 7/1000 inch) because the mixes are all fairly soft. Thus, differences in adhesion of different food preparations are not strictly comparable on a pound per square inch basis, but are closely comparable. If the purpose of the apparatus is to measure differences in adhesion which can be produced by altering a food or a tooth surface, then no such objection would exist.

If procedures involving a large number of teeth are contemplated, it is felt that cattle teeth could be used. The adhesion of foodstuffs to cattle and human enamel surfaces was similar. It seems important to use such biologic material rather than metal surfaces, because adhesion is known to depend on forces of intermolecular attraction and other surface phenomena such as "wetting" which are not likely to be reproduced on an artificial surface.

The difference, recorded in Fig. 2, between corn flake-water mixes and corn flake-saliva mixes is interesting. Recently, Swenander Lanke[10] has investigated the influence of certain properties of foodstuffs and individual oral conditions on the salivary sugar level. Among other factors which would influence sugar elimination from the mouth are the protein or gluten content of the cereal and the amylase content of the saliva. According to Swenander Lanke, a gluten rich bread would be retained less time than bread containing less gluten, and amylase rich saliva would favor a more rapid clearance of starch. Our measurements revealed that saliva produces a considerably less sticky mix with corn flakes than does water, and it seems that the amylolytic properties of saliva may be of considerable importance in reducing the adhesiveness of starch in the mouth.

At the present time, further work is in progress to improve the testing procedure and it is hoped that such a test procedure will be of value in considerations on the diverse phenomena associated with intraoral food retention.

SUMMARY

1. A method has been developed for measuring the adhesion of foodstuffs to tooth surfaces. This involves modification of a standard tensile strength tester to measure the strength of food-tooth bonds in pounds per square inch.

2. It is possible to compare the adhesiveness of a foodstuff under different conditions, and also to compare the adhesiveness of different foods.

3. Variations noted in the adhesiveness of foodstuffs on different test surfaces demonstrate the importance of using enamel surfaces in such measurements.

4. Alternative uses and limitations of the apparatus were reported.

The author wishes to acknowledge the cooperation of Southern Research Institute, Birmingham, Ala., in making the Scott I.P.-4 tester available for this study. Part time technical assistance was rendered by Osmund Appling Coleman and Bryant Gordon Speed, dental students.

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RESEARCH ANNOTATIONS*

DEVELOPMENT OF COATINGS FOR APPLICATION TO TEETH

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ONE approach to the prevention of dental caries is to form a protective coating on the tooth surface. Long chain aliphatic amines have been used for this purpose (Green, R. W., and Walsh, J. P.: J. D. Res. 30: 218, 1951). The use of silicones in dental practice has been reported (Phatak, N., Savara, B. S., and Dement, J.: J. D. Res. 34: 788, 1955, and Savara, B. S., Phatak, N. K., and Dement, J. A.: J. D. Res. 37: 24, 1958), and many coating materials show promise for dental applications (McGregor, R. R., Silicones in Industry, New York, 1954, McGraw Hill).

For a coating to be successful it should adhere to the enamel surface for a reasonable length of time and be resistant to wear, and to the action of salivary enzymes and their products. Coatings prepared from organic derivatives of silicone, phosphorus, and titanium were chosen as most likely to meet these requirements.

Materials and Methods.—The teeth used were lower incisors from cattle. The teeth were washed in water and cleaned by lightly brushing the surface with a slurry of pumice.

It was decided early that the tooth surface would have to be "primed" to produce an adhesive surface before the coating was applied.

Tetrabutyl orthotitanate was used as a primer in 2 per cent solution in V.M. & P. naphtha painted on the teeth and allowed to air dry for 5 minutes. In other attempts to obtain an adhesive surface, silicone adhesives were used. As suggested by Buonocore (J. D. Res. 34: 749, 1955), Eastman 910 adhesive (a cyanoacrylate monomer) was also used as a primer.

For coating the tooth surface many monomers and polymers were used. Only a few titanium and phosphorus compounds were available, so most effort was directed to investigations of silicone compounds.

To evaluate the coatings, the teeth were stored in water at room temperature for 2 months. The teeth were then examined for discoloration, peeling, or "tacking" of the coating. The water repellency of the coating was tested by observing the flow characteristics of a blue-black permanent ink across the surface. Satisfactory coatings were tested by comparing the adhesiveness of a food to a tooth surface before and after coating. A tensile strength measurement of food-tooth bonds was made (Caldwell, R. C.: J. D. Res. 38: 188, 1958) with mixtures of 200 mg. of corn flakes and 0.1 ml. of water.

Results and Conclusions.—Use of a primer prior to application of the protective coating improves the effectiveness of the applied coating. The most satisfactory primer was tetrabutyl orthotitanate. The silicone adhesives were unsatisfactory because subsequent coatings were tacky or peeled off the teeth. Application of a 2:1 mixture of Dow Corning 200 Fluid (350 centistokes) (a dimethylpolysiloxane), and Tennessee Eastman Chemical Products' 910 adhesive (cyanoacrylate monomer) resulted in a water-repellent, nonpeeling coating. Dow Corning 200 Fluid (350 centistokes) applied over tetrabutyl orthotitanate also gave a satisfactory result. Tensile strength results ranged from a 69 per cent reduction to 37 per cent increase in adhesion, but after 7 days in water the reduction in adhesion was no longer apparent.

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LYMPHATIC DRAINAGE OF TEETH IN DOGS DEMONSTRATED BY RADIOACTIVE COLLOIDAL GOLD

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THE presence of lymphatic drainage from the teeth has not been satisfactorily demonstrated by conventional methods. Fish (J. A. D. A. 14: 804-817, 1927) demonstrated the "circulation" of lymph in the dentin and enamel of dogs' teeth by the use of dyes and India ink.

In an attempt to investigate further the lymph flow in this area and its subsequent drainage by the lymph nodes, radioactive colloidal gold (Au^{198}) was placed in a cavity preparation extending into the dentin of a lower cuspid tooth. The preparation was then sealed with a low fusing wax. The radiogold was allowed to stay in the tooth for 4 hours. At the end of that time the left and right submandibular lymph nodes were removed, as were the cervical and inguinal lymph nodes. Assays for radioactivity were done by placing each of the nodes in vials which fitted into a scintillation counter. Since Au^{198} emits gamma rays, no additional sample preparation was necessary. The submandibular lymph node on the same side as the tooth in which the radiogold was placed contained detectable radioactivity four or five times above background. No detectable radioactivity was present in the other lymph nodes sampled, indicating that the colloidal gold did not enter the general lymphatic circulation in significant amounts. The amount of radioactivity present in the lymph node in relation to the total radioactivity introduced into the tooth represented less than 0.001 per cent. The use of the radioactive colloidal gold in this type of experiment enabled us to determine the extremely minute but significant transfer of the colloidal gold through the dentin via the lymphatics and hence to the submandibular lymph node.

These experiments were repeated in 8 dogs and positive results were obtained in 6 of the animals. Factors such as the physical occlusion of the dentinal tubules during the preparation and the age of the dog may be responsible for the two negative results.

Because of the short half-life of the Au^{198} and the relatively low radioactivity levels, the autoradiographic visualization of the pathway could not be determined. The localization of the radioactivity in the lymph node draining the tooth is presumptive evidence for lymphatic drainage of this structure.

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A METHOD FOR PRODUCING FLUORIDE-FREE SALTS AND A FLUORIDE-FREE DIET

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ATTEMPTS were made to remove fluoride from ordinary foodstuffs. For example: casein, precipitated from milk by centrifuging at 50,000 r.p.m., contained 1 ppm fluorine. By washing with fluorine-free, isoelectric water, this could be reduced to 0.2 ppm but not entirely eliminated.

Although water could be freed from fluorine by re-distillation from alkali, sulfuric and phosphoric acids could be freed from fluoride by adding water and boiling in glass or silica vessels, and sodium and potassium salts could be freed from fluoride by repeated recrystallizations. This was not possible with calcium salts. Reagent grade calcium salts contained about 1 to 6 ppm of fluorine which accompanied the calcium through all precipitations and crystallizations. It was found, however, that in the precipitation of calcium phosphate (hydroxyapatite), the first precipitate formed was higher in fluorine than that formed later; in fact, the last of the precipitate to come down was fluoride-free, since all the fluoride in the solution had been used in forming fluorapatite of the hydroxyapatite first formed.

Three-tenths gram molecule of reagent calcium chloride was dissolved in water to make 1 L. and 0.2 gram molecule of trisodium phosphate in a similar liter. The sodium phosphate solution was stirred into the liter of calcium chloride solution and the precipitate of hydroxyapatite was separated and placed in a column with filter at the bottom. The fluoride was removed from solutions of various salts by passing through this column.

Since so many chemical compounds are needed to make up a synthetic diet and since I failed in removing the fluorine from the first tried (casein), I grew food in fluoride-free, nutrient solution. Rain water caught on an aluminum roof 10 miles from the nearest open hearth furnace and 70 miles from the sea contained 0.002 to 0.004 ppm of fluorine. Several different brands of anion exchangers were set up and the rain water passed through them, the Amberlite IR[4] removing the most fluorine. This was then treated with 4 per cent aluminum chloride and washed with distilled water until the washings were free from chloride. When the rain water was passed through a 20-inch column of this aluminum-treated Amberlite IR[4], no fluorine could be detected in it.

The method of Shive and Robbins (*N. J. Agric. Exper. Sta. Bull.* 636: 5, 1942) was used to produce fluoride-free, hand pollinated, yellow corn by removing the fluorine from each ingredient of the nutrient solution. Yeast was grown in a decoction of fluoride-free corn stalks and additives in fluoride-free water.

Ten-gram samples of the corn, yeast, and a complete diet were burned in a platinum tube and analyzed by the method of McClendon and Foster (*Indust. & Engin. Chem.* [*Anal. Ed.*] 13: 280, 1941). This method showed an error of 7 per cent in analyzing 1 μg of fluorine added to 5 grams of diet. In analyzing water, 100 mg. of fluorine-free sodium hydroxide was added to 1 L. of water. It was evaporated to dryness and the residue analyzed.

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JAPANESE SECTION
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PROCEEDINGS OF THE SIXTH ANNUAL MEETING
NOVEMBER 1, 1958

FACULTY OF DENTISTRY, OSAKA UNIVERSITY, OSAKA, JAPAN

1. PHOTOELASTIC STUDIES ON MAXILLARY AND MANDIBULAR BONES.—*Haruo Murata, Matsukichi Ueyama, Harutaka Imada, Sanya Sakada, and Mamoru Yamada, Dept. of Physiology, Faculty of Medicine, Tottori University.* The two-dimensional distributions of principal and boundary stresses in the maxillary and mandibular bones on an occasion of normal occlusal movement were investigated by using the photoelastic method in order to make clear the cause of pyorrhea.

In the previous meeting, one of the authors reported the boundary stresses of mandibular bone. In this report, the principal stresses were investigated. The results showed the stresses were not at the alveolar border or alveolar ridge in each direction of applied forces at the incisor, canine, premolar, or molar teeth. Compression stresses and singular points were shown at the apex area of alveolar sockets.

In the maxillary bone, the principal and boundary stresses were examined. The boundary stresses of each tooth indicated the minimum value when occlusal movements were in the normal direction, but it had a tendency to increase when pressure was added from buccal or labial sides. Principal stresses of this bone showed the same results as with the mandibular bone.

From these results, the authors suggested the improbability of mechanical force as an important cause of pyorrhea.

2. FACIAL GROWTH AND DENTAL DEVELOPMENT IN OBESE CHILDREN.—*Naoto Kawakami, Dept. of Orthodontics, Nippon Dental College.* So-called obese children are relatively few among the Japanese. Two cases of obese children, whose dental development and facial growth have been observed for 6 years, are worth reporting. Their dental developmental stages show noticeable advancement and the facial growth exceeds, in every dimension, the standards of Japanese children with normal occlusion.

It is interesting to note that only one feature in facial depth (auriculonasion) was within normal range and progressed normally, while all other features in all dimensions showed extremely supernormal growth.

3. ROENTGEN CEPHALOMETRIC STUDIES ON THE GROWTH OF DENTOFACIAL COMPLEXITY OF JAPANESE CHILDREN.—*Tetsuo Iizuka, Dept. of Orthodontics, Tokyo Medical and Dental University.* The purpose of this study was to obtain a criterion—roentgen cephalometrics—of the changes of dentofacial complexity of growing Japanese children. The author will attempt to utilize this criterion in the case analyses of Japanese orthodontic patients. Subjects used for this study numbered 232 were selected from 1,650 boys and girls, and had normal occlusion. Ages ranged from 4 to 12 years, and for the convenience of this study the

subjects were divided into 6 groups (IIA, IIC, IIIA, IIIB, IIIC, and IVA)—according to Hellman's dental age. Twenty-three angular and 20 linear measurements were made on all of the subjects and the results of the measurements were statistically treated, that is, means, standard deviations, and probable errors were derived for each group.

The following results can be mentioned as the most interesting and valuable findings.

a. The angle of the facial plane, Y-axis, and mandibular plane to the SN plane, and gonial angle were extremely stable, showing no noticeable change through all stages.

b. The quadrilateral (NMGZ) of each increased equally in all directions, so that the figure of NMGZ was little influenced by growth.

c. The angle of the upper incisor axis to the FH plane and SN plane increased markedly between IIC and IIIB.

d. The angle of the lower incisor axis to the mandibular plane increases markedly between IIC and IIIB.

e. N-S distance, the basic plane of the cranium, increases in each successive stage, showing the most significant increase between IIIA and IIIC.

f. There is a significant increase in the height of the anterior and posterior face (N-M, G-Z), although the growth of the anterior face was larger than that of the posterior.

4. The Electrical Responses of the Various Sensory Terminals in the Dental Pulp, Periodontal Membrane, and Gingivae.—*Sanya Sakada, Hirotake Tamoto, Sentaro Kumano, Haruo Murata, Matsukichi Ueyama, Harutaka Imada, and Mamoru Yamada, Dept. of Physiology, Faculty of Medicine, Tottori University.* For a long time, a greater interest has been shown in the morphology of the sensory terminals of the dental nerve than in the physiology thereof. Several researchers, however, have studied the physiologic characteristics of excitation by electrical recording of the sensory terminals in the dental pulp and the periodontal membrane. These characteristics, moreover, were reinvestigated by electrical, mechanical, thermal, and chemical stimuli.

Dental pulp (cat, dog): It was observed that responses were not recorded in the incisor nerve (which innervates the dental pulp) by application of mechanical stimulus. When enamel, dentin-enamel, dentin, or predentin were stimulated by electrical stimulus, threshold and rheobase were measured and, at the cervical part, the thresholds of electrical, thermal, and chemical stimuli were elucidated.

Periodontal membrane (rabbit, dog): A burst of impulses or several impulses were recorded from the mandibular nerve when a rapid mechanical stimulus was applied to the incisor or canine crowns. Occasionally, a spontaneous discharge resembling that reported by Ness (1954) was observed, but it was thought to be due to the effect of operation.

Gingiva (rabbit): The sensory terminals of touch, pain, cold and warmth were found, and their thresholds were examined.

5. Influences of Zinc Iontophoresis Used for Dentin Hyperesthesia Upon Dentin and Pulp.—*Masaomi Inoue and Kensaku Suzuki, Dept. of Operative Dentistry, Tokoyo Medical and Dental University.* In order to investigate the mechanism of zinc iontophoresis, which showed a markedly favorable effect in dentin hyperesthesia, the permeability of zinc ions into the dentin and the

histopathologic changes in the pulp were examined after application. The selected materials were extracted human teeth and human teeth in situ. The results follow.

a. Zinc iontophoresed from the surface of exposed dentin was deposited in the dentin beneath the exposed area as an insoluble zinc compound.

b. Clear and substantial changes were noted in the dentin, which changes corresponded with the pathway of electricity directly from the surface of exposed dentin to the pulp.

c. It was presumed that the above-mentioned changes were caused by the fact that calcium in the dentin was replaced by zinc.

d. In an application of more than 1 Ma. per minute, the vacuolization at the odontoblast layer was observed. This change might be interpreted as the movement of water into the pulp from the surface of the tooth. Some histologic figures showed that the water passed through the space between the dentinal tubules and odontoblastic processes.

e. In an application of less than 0.8 Ma. per minute, a markedly favorable effect on inflamed pulp was noted, although histologic changes were very few.

6. On the Transference of Enamel Matrix Substance to the Dentin and the Dental Sac.—*Syoichi Suga, Dept. of Pathology, Nippon Dental College.* It was previously reported that the stain-color of acid-insoluble enamel matrix—by Weigert's resorcin-fuchsin stain, was the same as that produced in elastic fiber by various shaping substances. Using this knowledge, one can identify cells in the intra- and intercellular portions of the ameloblasts and stratum intermedium.

One of these resorcin-fuchsin stainable substances is the protein containing the SH group. These substances may correspond to so-called keratohyalin, eleidin, and keratin, or similar substances, such as the epithelial products.

It is supposed that these resorcin-fuchsin stainable substances are secreted from the ameloblasts and the stratum intermedium cells, and are transferred to the enamel matrix at each stage.

On the other hand, it has been found that, in various animals, such substances as these transfer and penetrate to the dentinal matrix and tubules from the side of the enamel organ and enamel matrix. In rats, these substances penetrate to the matrix and tubules in the superficial zone of the dentin, at the pre-formative stage, and penetrate deeply to the dentinal tubules, at the end of the formative stage and at maturation. In guinea pigs, they penetrate to the superficial dentinal matrix from the pre-formative stage to the formative stage, and also penetrates to dentinal tubules or, frequently, even to the odontoblastic layer through dentinal tubules. In pigs and men, they penetrate deeply up to the dentinal tubules, at the first part of the formative stage but, in dogs, their penetration is rather shallow at this stage.

The above-mentioned findings suggest the following conclusions.

a. Dentin plays an important role in enamel matrix formation.

b. Part of the enamel matrix substances secreted from the ameloblasts and stratum intermedium is in a fluid state, at least enough so as to be able to penetrate to the enamel matrix (which has already formed), the dentinal tubules, and the dentinal matrix.

c. The penetration of these substances may be due to the circulation of the tissue fluid which has come from the enamel organ to the dental pulp.

d. The transference of these substances to the dental sacs, which has been observed in guinea pigs, may suggest the reversal of circulation of tissue fluid accompanying the completion of matrix formation therein.

7. Three-Dimensional Photoelastic Studies on the Clasp-Rest and Tooth Extraction.—*Hiroshi Ito, Yoshio Inoue, and Mamoru Yamada, Dept. of Physiology, Faculty of Medicine, Tottori University.* The effects of clasp-rests on abutment teeth and on the extraction of teeth from the alveolar bone were determined by a three-dimensional photoelastic experimental method. The photoelastic sensitive material used was the epoxi resin, Epikote, and other resins. The three-dimensional experimental procedures were as follows: after engraving each half of the pulp cavity in two pieces of cubic plate of epoxi resin, these pieces were pasted over each other with the same resin and were carved in the shape of a tooth. One of various types of rests was equipped with one of each model tooth. Pressure was applied to the stem of the clasp and on teeth adjacent to the extracted one and, at the same time, stresses in the models were fixed or frozen.

The effects upon the various rests indicated the superiority of the spoon-shaped one. When teeth were extracted, stresses resulting from the three-dimensional experiments were found in the margin and apex areas of the alveolar socket, with the lines of stress declining to the extracted side in contrast to the normal state. On the basis of the two-dimensional results, the authors discussed the probability of Sandstedt's "Compression Theory" and Oppenheim's "Transformation Theory" in their relation to these problems.

8. Minute Crescent-Shaped Organisms Isolated From Dental Scrapings.—*Masao Onisi and Teiji Kato, Department of Microbiology, Tokyo Medical and Dental University, School of Dentistry.* Characteristic lobate colonies of Vibrio were recognized on the Kasai's medium seeded with dental scrapings during 3 days' incubation at 37° under anaerobic conditions. Fifteen isolates from 4 specimens, stained negatively by the gram method, were all small, strongly curved, comma-shaped organisms, moving actively with tufty flagella attached to the concave side near one end. They translocated at times like cholera vibrios and sometimes moved rotatively. On solid media, containing 3 per cent agar, they grew into smooth, convex colonies. They produced neither gas nor putrefactive or disagreeable odor during growth in glucose or other broth. Nitrate was reduced. There was no production of indol or H_2S. No coagulation of the litmus milk was found, but six isolates reduced litmus. There was no liquefaction of gelatin. From glucose they produced nongaseous acid sufficient to decrease the pH of glucose broth to 5.3, but there was no acid from maltose, lactose, sucrose, glycerol, or starch. There was neither greening nor hemolysis on the blood agar plate. Intraperitoneal injections of cell suspension in guinea pigs did not cause any significant pathologic changes.

The present isolate, thus resembling one species, coincides with neither asaccharolytic *V. sputorum*, which grows only on media enriched with serum and produces H_2S, according to Bergey's Manual, nor larger sized *V. buccalis*, which ferments lactose and sucrose, according to Prevot. It also does not resemble *Selenomonas sputigena* with respect to size, the place of attachment of the flagella, or activity of carbohydrate fermentation. A species similar to the present isolate is small-sized *V. stomatidis*, which moves rotatively, according to Prevot. This species, however, is virulent to experimental animals and ferments glucose and lactose.

9. Rotation of Maxillary Central Incisors.

A. A Wing-Like Appearance of Upper Incisors Among American Indians.—*A. A. Dahlberg, Research Associate (Associate Professor), Zoller Memorial Dental Clinic and Department of Anthropology, University of Chicago.* (Paper read in absentia.) A wing-like appearance of the incisor teeth is frequently encountered in some hominoid populations. This is due to the rotated

position of these teeth in their sockets. The distal part of the crown is turned forward and the mesial part is in a lingual position. This condition occurs unilaterally as well as bilaterally.

The reverse position to the mesiolingual rotation also occurs in varying frequency with a higher incidence in groups having high and narrow palates. This counter-winged position gives the impression of a rather pointed prominence of the upper incisors.

The Indian dentition cast collections of the Zoller Laboratory of Dental Anthropology offered a large sample of several groups for a systematic study of the anterior tooth positions. Data on the positions of 1,825 Amerinds from ten different tribes were recorded and compared to a sample of 200 Chicago Caucasoids. The frequencies of the different categories of tooth position varied from tribe to tribe. These in turn were markedly different from that of the Caucasoid.

Five classes of position were used in the study. These included all teeth in straight alignment, bilateral winging, unilateral winging, bilateral counter-winged position, and unilateral counter-winging.

A higher frequency (38 per cent) of bilateral winging was found in the Maricopas than in the other groups, who ranged from 22 to 29 per cent. All the groups had a large number whose anterior teeth were aligned straight in the normal arch curves. These ranged from 29 per cent in the Zunis to 49 per cent in the Pimas. Counter-winging was very low among the Pima (3 per cent). The Pine Ridge Sioux exhibited this tooth position in 28 per cent with other groups having intermediate frequencies. Only 3 per cent of the Caucasoid group showed the winging, but 42 per cent showed counter-winging.

The reason for the difference between the Amerind and Caucasoid anterior tooth positions is not clear. Uncrowded dentitions in both groups are to be found exhibiting the positions mentioned. Anatomic patterns of the lingual surfaces may account for some of the variations. The arch form and vault height no doubt influence these positions to some extent. Pedigree studies show a consistent similarity of this trait in families, which evidence points to a genetic cause.

B. Bilateral Rotation (Mesiopalatal Torsion) of Maxillary Central Incisors.—*Kei Enoki and Elaiko Nakamura, Dept. of Orthodontics, Nippon Dental College.* Bilateral rotation of the maxillary central incisors is frequently seen clinically among orthodontic patients in Japan. The present study was carried out to find the frequency of this phenotype among Japanese children and its relation to occlusion, dental arch development, and facial growth. Bilateral winging was seen in 9.64 per cent of 1,089 school children—7 per cent of males and 12 per cent of females.

Various "width" factors, such as width of incisors, space available for those two incisors, coronal and basal arch width, and facial width (bizygomatic and bicondylar) were measured.

Since no positive relationships were found between this phenotype and the above "width" factors, it can be said that the genesis of this feature is not due to local factors or growth. That this feature has a significant racial distribution has not yet been demonstrated.

10. Regrowth of Capillary Vessels on the Gingival Wound.—*Yoso Imagawa, Dept. of Operative Dentistry, Tokyo Medical and Dental University, and Kazuo Akita, Dept. of Oral Surgery, Okayama University.* (No abstract submitted.)

11. Casting Shrinkage of Gold and Alloys.—*Takao Fusayama, Dept. of Operative Dentistry, Tokyo Medical and Dental University.* Many researchers have reported different findings on the casting shrinkages of gold and gold alloys. Two of the widely accepted values are those of 1.5 per cent for 22-carat gold by Hoolenback and Skinner (1-inch specimen) and 1.25 per cent for 90 per cent gold-copper alloy (3-inch specimen by Coleman). Although either of these figures may be accurate, it is inadvisable to calculate the desired investment expansion from them since the value of casting shrinkage may be dependent on the size and form of specimens.

In this study, four different types of casting specimens, having sizes and forms comparable to practical inlays or crowns were used, and the following values for casting shrinkage were obtained with gold alloys: (1) 2.1 per cent in simple inlays, (2) 2.0 per cent in crowns, and (3) 1.9 per cent in MOD inlays.

12. On the Dilating Effects of Pulp Anodynes on Blood Vessels.—*Taro Yao and Nobuhiko Takagi, Dept. of Pharmacology, Osaka Dental College.* (Read by title only.)

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